The Role of Nogo-A in Tooth Development and Regeneration

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1. Summary

Initially discovered as a potent inhibitor of neurite outgrowth, in the last decades Nogo-A emerged as a fundamental actor of nervous system development, physiology, pathology and regeneration. In this context, Nogo-A is emerging as a promising target for central nervous system (CNS) regeneration, in particular following spinal cord injury and stroke. Moreover, Nogo-A was identified as a major regulator of cell adhesion/migration within the CNS. However, despite the already known widespread expression of Nogo-A in non-neuronal tissues, its role outside the CNS has been poorly investigated.

Teeth are the most studied organs of the orofacial complex and represent an extremely powerful model for studying the development and regeneration of ectodermal organs. In particular, the continuously growing mouse incisor represents a great tool for studying the physiology of epithelial and mesenchymal stem cells, as well as the molecular mechanisms underlying the development of mineralized tissues.

In this work we investigated the expression and the roles of Nogo-A in mouse tooth development. We found that Nogo-A is expressed in the developing teeth. Using a mouse genetic model, we observed that the deletion of Nogo-A (Nogo-A KO) leads to significant alterations in dental epithelial cells and their structures that result in enamel defects *in vivo*.

Transcriptome analysis conducted on the lower incisors of Nogo-A KO mice revealed that Nogo-A deletion affects both the dental epithelial stem cells niche and the differentiated region of the incisor, thus suggesting that Nogo-A might play multiple roles during the various processes of tooth development.

Mouse incisors treated with a Nogo-A neutralizing antibody *in vitro* exhibited an increased growth of the dental epithelium. This was the effect of the increased proliferation of both epithelial and mesenchymal stem/progenitor cells at the cervical loop area of the incisors after the blocking of Nogo-A function.

Co-cultures of trigeminal ganglia and developing tooth germs revealed that Nogo-A deletion in teeth could alter their repulsive/attractive effects on the growing nerve fibres.

Taken together these results show that Nogo-A plays an important role in tooth development and in the regulation of dental stem/progenitors cells and, moreover, indicate a completely novel group of targets for Nogo-A signalling outside the CNS. It is thus possible that Nogo-A has similar roles in other organs, most particularly as a fine regulator of stem/progenitor cells proliferation and differentiation.

2. Zusammenfassung

In den letzten Jahrzehnten konnte gezeigt werden, dass Nogo-A, das ursprünglich als potentieller Inhibitor von Neuritenwachstum entdeckt wurde, in der Entwicklung, Physiologie, Pathologie und Regeneration des Nervensystems eine fundamentale Rolle spielt. In diesem Zusammenhang wird Nogo-A als ein vielversprechendes Molekül für die Regeneration des Zentralen Nervensystems (ZNS), vor allem nach Rückenmarksverletzungen oder Schlaganfällen, gehandelt. Darüber hinaus regelt Nogo-A die Adhäsion und Migration von Zellen im ZNS. Trotz der bereits bekannten Expression von Nogo-A in nicht-neuronalen Geweben, verbleibt seine Rolle außerhalb des ZNS bisher weitreichend unbekannt.

Der Zahn ist das am besten untersuchte Organ des orofazialen Komplexes und daher äußerst interessant für die Erforschung der Entwicklung und Regeneration ektodermaler Organe. Dabei stellt vor allem der stetig wachsende Schneidezahn von Mäusen ein hervorragendes Modell zur Untersuchung der Physiologie von epithelialen und mesenchymalen Stammzellen, sowie der molekularen Mechanismen, die der Entwicklung mineralisierter Gewebe zu Grunde liegen, dar.

In dieser Studie haben wir die Expression von Nogo-A wie auch die Rolle, die Nogo-A während der Entwicklung von Mauszähnen spielt, genauer untersucht und konnten zeigen, dass Nogo-A während der Zahnentwicklung in Mäusen exprimiert wird. Mit Hilfe eines Mausmodells, bei dem Nogo-A deaktiviert wurde (Nogo-A KO Mäuse), konnten wir beobachten, dass das Fehlen von Nogo-A zu signifikanten Veränderungen in dentalen Epithelzellen und derer Strukturen führt, was in vivo Defekte des Zahnschmelzes nach sich zieht.

Eine Transkriptomanalyse der Unterkieferschneidezähne von Nogo-A KO Mäusen ergab, dass der Verlust von Nogo-A sich sowohl auf die dentale Epithelstammzellnische als auch auf die differenzierte Region des Schneidezahns auswirkt. Dies zeigt, dass Nogo-A möglicherweise mehrere Funktionen während der Zahnentwicklung hat.

Mausschneidezähne, die in vitro mit neutralisierenden Nogo-A Antikörpern behandelt wurden, wiesen ein verstärktes Wachstum des dentalen Epithels auf. Zurückzuführen war dies auf eine Zunahme der Proliferation von Epithel- als auch von mesenchymalen Stammzellen in der Stammzellnische der Schneidezähne durch die Blockierung von Nogo-A.

Kokulturen von Trigeminalganglien mit Zahnkeimen zeigten, dass die Deaktivierung von Nogo-A in Zähnen deren Wirkung auf das Verhalten von wachsenden Nervenfasern verändert.

Zusammengenommen verdeutlichen diese Ergebnisse die Bedeutung von Nogo-A während der Zahnentwicklung und bei der Regulierung von dentalen Stammzellen und deuten auf eine völlig neue Gruppe von Targets für den Signalweg von Nogo-A außerhalb des ZNS hin. Daher ist es möglich, dass Nogo-A in anderen Organen ähnliche Funktionen, vor allem bei der Stammzellproliferation und -differenzierung, hat.

3. Introduction

The Tooth: an overview

Teeth are the most studied organs of the orofacial complex and their early developmental stages closely resemble those of other epidermal appendages such as hair follicles and salivary glands (Jiménez-Rojo et al., 2012). Although teeth show an incredibly high diversity across species, they all share common anatomical features.

The tooth is composed by a combination of mineralized and soft tissues. Enamel is a hard, inert, highly mineralized acellular tissue, which forms the crown of the tooth and directly mediates mastication. A less mineralized cellular hard tissue, the dentin, which is formed by specialized cells of the dental pulp, supports the enamel. The dental pulp is a soft connective tissue that conveys innervation and vascularization (figure 1). The tooth is attached to the alveolar bone of the jaw via the tooth root that is composed by hard (dentin and cementum) and soft (periodontal ligament) tissues that ensure the right balance between firm adhesion and mechanical flexibility of the tooth (figure 1).

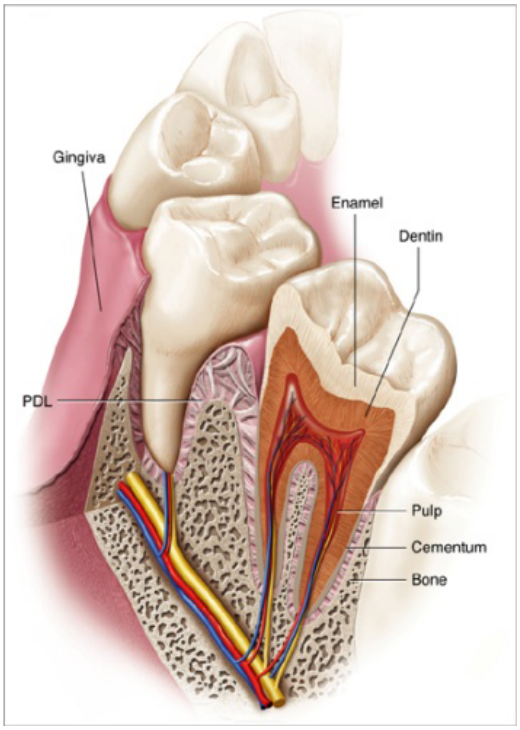


Figure 1. General structure of human teeth. (Nanci, 2013)

Orofacial and tooth development

The orofacial complex originates from four processes: the frontal, the mandibular, and the two maxillary processes. Each process comprises layers of epithelial, mesodermal, and cranial neural crest-derived cells (CNCCs). CNCCs originate from the dorsal edges of the folding neural plate. During their migration, CNCCs intermingle with the paraxial mesoderm, forming the mesenchyme of the facial prominences. The mesoderm gives rise to the jaw musculature, while CNCC-derived mesenchyme forms the cartilage, connective tissues, bones and all organs (e.g. teeth and salivary glands) of the orofacial area (Minoux and Rijli, 2010; Pagella et al., 2015b; Mitsiadis and Luder, 2011). The development of these organs is based on reciprocal interactions between epithelial and mesenchymal cells (Handrigan et al., 2007; Mitsiadis and Graf, 2009; Pagella et al., 2015b). Inappropriate signalling can alter these tissue-tissue interactions, resulting in orofacial malformations that account for approximately one-third of all birth defects (Dixon et al., 2011; Kouskoura et al., 2011).

Tooth development has been mainly studied in mice. The initial stages of mammalian tooth development proceed through a series of well-defined morphological stages (Mitsiadis and Graf, 2009). The dental epithelium originates from the first branchial arch and a smaller part of the frontonasal process (Cobourne and Mitsiadis, 2006; Pagella et al., 2015b; Mitsiadis and Luder, 2011). Odontogenesis starts with epithelial thickenings, called the *dental placodes*, at the sites of the future dental arches in the maxilla and mandible at mouse embryonic day 10.5 (E10.5). The dental epithelium undergoes successive morphological changes that can be divided into stages according to their morphological appearance (figure. 2)

The dental placode invaginates into the underlying condensing mesenchyme and forms an epithelial *bud* (bud stage, E12.5-E13.5 in mice, figure 2). At this stage a cluster of non-dividing epithelial cell appears at the tip of the bud, called the *primary enamel knot*, which acts as a signalling centre that guides further tooth morphogenesis (Jernvall et al., 1994; Mitsiadis and Graf, 2009). Continuous proliferation and growth of the dental epithelium around the condensed mesenchyme leads to an epithelial *cap* configuration (cap stage, E14.5-E15.5 in mice, figure 2). This epithelial outgrowth, which will give rise to the enamel, is generally referred to as *enamel organ*. The underlying engulfed mesenchyme, which will form the pulp and the dentin, is called *dental papilla*. The ectomesenchyme that encapsulates the enamel organ and will give rise to the supporting tissues of the tooth is called *dental follicle* (Nanci, 2013). At the cap stage three different epithelial layers start to appear: the *stellate reticulum* (SR), the *inner enamel epithelium* (IEE) and the *outer enamel epithelium* (OEE). The SR, which is located between the IEE and the OEE, is composed of star-shaped epithelial cells connected to each other as well as with the IEE and the OEE cells through desmosomes. The IEE faces the dental mesenchyme, while the OEE faces the dental follicle (figure 2). At the meeting point of IEE and OEE the *cervical loops* are formed.

At a more advanced developmental stages the enamel organ acquires the *bell* configuration (bell stage, E16.5-E18.5 in mice, figure 2). Additional enamel knots, named *secondary enamel knots*, start to be formed in the IEE that correspond to the future cusps of the molars and give the specific tooth crown morphology (Jernvall et al., 1994). At the tip of the cusps, mesenchymal cells start to differentiate into *odontoblasts* that produce dentin, while shortly after IEE cells facing the odontoblasts start to differentiate into the enamel-secreting *ameloblasts* (figure 2) (Mitsiadis and Graf, 2009). Cells of the *stratum intermedium* (SI) are localized on top of IEE cells that form the ameloblastic layer. Ameloblasts and all other epithelial cell populations participate in enamel maturation and form the reduced enamel epithelium of the crown that is lost when the tooth erupts into the oral cavity.

Cytodifferentiation and hard tissue deposition proceed in the cervical (apical) direction (for dentin and enamel development, see section "Development of dental mineralised tissues: dentin, enamel"). Crown formation is followed by the development of the tooth root, which results in the initiation of tooth eruption. The crown-to-root transition begins at the cervical loop areas where IEE and OEE form a bilayer termed *Hertwig's epithelial root sheath* (HERS). The HERS that guides the root development is progressively fragmented and gives rise to the *epithelial rests of Malassez* (ERM). ERM are dental epithelial cell remnants within the periodontal space that regulate homeostasis and regeneration of the periodontal space. Epithelial cells from the HERS give rise to *cementoblasts* that produce the *acellular cementum* of the root, while mesenchymal cells of the dental follicle give rise to cementoblasts that form *cellular cementum* of the root (figure 1) (Catón et al., 2011; Nanci, 2013).

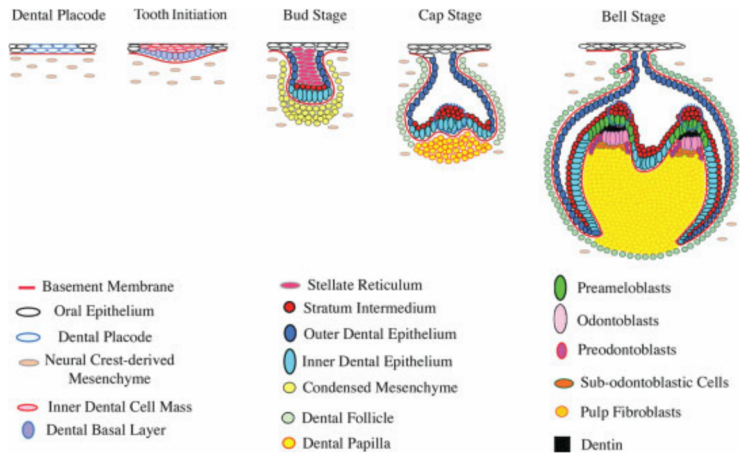


Figure 2. Main stages of tooth development (Mitsiadis and Graf, 2009)**.**

Development of dental mineralized tissues: dentin, enamel

A key step in tooth development is by the formation of dentin and enamel. Dentin is the mineralized tissue that constitutes the bulk of the tooth and is intimately connected to the dental pulp (Arana-Chavez and Massa, 2004). Deposition of dentin starts at the onset of odontoblasts differentiation. Newly polarized odontoblasts emit short processes from the surface facing the preameloblasts. Differentiating odontoblasts

secrete a matrix mainly composed by collagen fibrils, called mantle dentin. As differentiation proceeds, one of the processes of each odontoblast gradually becomes accentuated and constitutes the process around which dentin matrix mineralizes (Arana-Chavez and Massa, 2004). As a result of the presence of these processes, the dentin is crossed by thousands of dentinal tubules, connecting the pulp with the dentinoenamel junction. With the stabilization of the processes and the formation of tight junctions between the odontoblasts, the secretion of non-collagenous matrix proteins begins. The dentin secreted in this phase, called circumpulpal dentin, shows thinner collagen fibrils and is rich in non-collagenous proteins such as dentin sialophosphoprotein (DSPP) , dentin matrix protein-1, -2 and -3 (DMP-1, DMP-2, DMP-3) and small amounts of proteins also present in the bone matrix (osteopontin, bone sialoprotein, osteonectin and osteocalcin) (Arana-Chavez and Massa, 2004; Papagerakis et al., 2002; He et al., 2003). The dentin formed by secretion of collagen and non-collagenous proteins is called *intertubular dentin*. In addition, each odontoblast continues to secrete a non-collagenous matrix: this matrix mineralizes quickly between the previously formed intertubular dentin and the odontoblastic processes, constituting the *peritubular dentin*. The dentin formed up to the completion of root development is called *primary dentin*. Throughout life, odontoblasts continue depositing dentin, called *secondary dentin*. Moreover, in response to damages to the dentin and the pulp, odontoblasts can secrete the so-called *tertiary dentin* (Arana-Chavez and Massa, 2004).

Enamel development starts just after the beginning of dentin mineralization at the *dentino-enamel junction* (DEJ). At this stage, differentiating *preameloblasts* extend cytoplasmic projections through the basement membrane that separates them from the dentin matrix. During the *secretory stage*, preameloblasts differentiate into *secretory ameloblasts*. These cells have a columnar, highly polarized shape that ends, at the apical side, into a Tomes' process, a conical structure that points towards the forming enamel. Through this process, ameloblasts secrete the enamel proteins; all ameloblasts within the same row secrete from the same side of their Tomes' process. Secretory stage enamel is rich in proteins and therefore has a soft consistency (Bartlett, 2013). As they move away from the dentin surface, ameloblasts secrete large amounts of enamel and the enamel layer thickens. Associated to enamel proteins, mineral ribbons form rapidly perpendicular to the secretory surface of ameloblasts. These ribbons will eventually form a rod/prism, where ameloblast will create only one prism. During this secretory stage, ameloblasts not only move away from dentin as the enamel thickens, they also move in groups that slide by one another: this movement results in the peculiar decussating enamel prism pattern observed in rodent incisors or the entwined gnarled prism pattern seen in molars (Bartlett, 2013). The extreme hardness and the mechanical properties of enamel strictly depend on the precise spatiality of the rods and of the inter-rod enamel. Slight perturbations of this process can lead to defective enamel formation. Ameloblasts secrete three presumed structural proteins, amelogenin (AMELX), ameloblastin (AMBN) and enamelin (ENAM), as well as the matrix metalloproteinase 20 (MMP20).

After secretion (*transition phase*), ameloblasts turn into shorter maturation cells that release a final coating of aprismatic enamel. During the successive *maturation phase* ameloblasts secrete kallikrein-related peptidase-4 (KLK4) to remove the previously secreted proteins, while rod and interrod mineral crystallites expand in volume. Mature enamel is the hardest tissue of the human body, containing less than 1% of organic material, composed mainly of hydroxyapatite and low amounts of carbonate, sodium, magnesium and iron (Bartlett, 2013). The extraordinary mechanical properties of enamel depend on the precise temporal and spatial coordination of the different processes involved in its development. Even minor variations in this process can lead to the formation of different forms of defective enamel in humans. These clinical manifestations are grouped under the name of Amelogenesis Imperfecta (Crawford et al., 2007).

Molecular bases of tooth development

Tooth development is driven by epithelial-mesenchymal interactions. Classical tissue recombination experiments have shown that the molecular dialogue between dental epithelium and dental mesenchyme starts when the CNCC-derived cells contact the oral epithelium. These same experiments demonstrated that the initial inductive signal for tooth formation resides in the epithelium. Tooth formation was induced when the oral epithelium from mouse embryos (E9 and E11.5) was separated from the mesenchyme and cultured with non-dental mesenchyme (Mina and Kollar, 1987). After the formation of the dental placode (E11.5), the inductive potential shifts to the underlying mesenchyme. The dental mesenchyme is now able to induce tooth formation when cultured with epithelium of non-dental origin (Kollar and Baird, 1970; Mitsiadis and Graf, 2009). From this stage, the mesenchyme controls the advancing morphogenesis of the tooth (Kollar and Baird, 1969; Mitsiadis and Graf, 2009). Reciprocal interactions between the epithelium and mesenchyme also guide the later stages of tooth development involving the production of dentin and enamel (Bartlett, 2013).

Specific gene networks regulate all stages of tooth development. Several genes act at specific stages and in specific sub-regions of the developing tooth to regulate tooth patterning and cytodifferentiation events. The molecules involved in tooth development belong to well-conserved signalling pathways that regulate the formation of most mammalian organs, such as Wnt, Shh, Bmp (Bone morphogenetic protein), Fgf (Fibroblast growth factor) and Notch.

The first molecular signature of tooth development is the expression of *Pitx2* in the presumptive dental epithelium (E8.5 in mouse), which becomes restricted to the epithelium of the tooth germs at later developmental stages (Mucchielli et al., 1997; Mitsiadis et al., 1998b). During tooth morphogenesis, the Bmp, Shh, Wnt and Fgf signalling pathways are repeatedly required to drive different stages of development. Multiple members of these families of proteins are expressed already in the presumptive dental epithelium, and the blocking of any of these pathways results in the arrest of tooth development at

early stages (Mitsiadis and Graf, 2009; Lan et al., 2014). Conversely, over-activation of Fgf signalling, via the overexpression of Ectodysplasin (Eda)(Häärä et al., 2012) or the deletion of members of the Sprouty family (Klein et al., 2006; Lagronova-Churava et al., 2013; Charles et al., 2011), leads to the formation of supernumerary teeth. During these first phases of tooth development, the oral cavity undergoes intensive compartmentalization (figure 3). The combination of the expression of different signalling molecules and transcription factors provides a genetic code of the oral cavity that maps the position of the different classes of teeth (Mitsiadis and Luder, 2011).

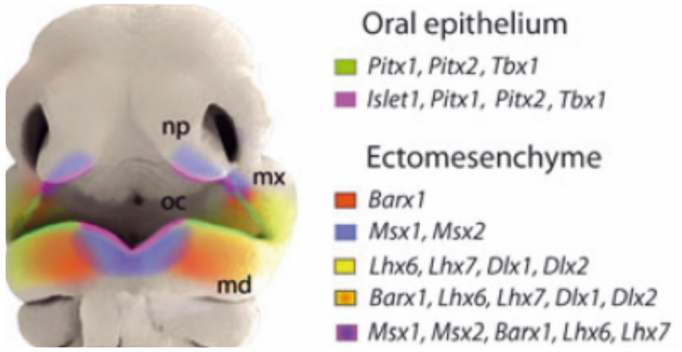


Figure 3. Molecular basis of tooth compartmentalization (Mitsiadis and Luder, 2011)

In this context, Bmp4 and Fgf8 play an important role. Epithelial-derived Bmp4 induces the expression of *Msx1* and *Msx2* in the underlying mesenchyme, which specifically lead to the development of incisors. In contrast, epithelial Fgf8 induces the expression of *Barx1*, *Dlx1*, *Dlx2*, *Lhx6* and *Lhx7* , all genes linked to the molariform shape of the tooth (Mucchielli et al., 1997; Mitsiadis and Luder, 2011). Bmp4 expression in the epithelium of the incisors is stimulated by *Islet1*, a transcription factor exclusively expressed in the anterior part (incisor field) of the oral epithelium. Ectopic expression of *Islet1* in the molar area stimulates the expression of *Bmp4*, which in turn stimulates *Islet1* and *Msx1* expression and inhibits the expression of *Fgf8* and *Barx1,* impairing molariform development (Mitsiadis et al., 2003). The tooth pattern specification also differs in the maxillary and mandible processes: deletion of both *Dlx1* and *Dlx2* impair the development of maxillary molars, while deletion of *Pitx1* results in misshaped mandibular molars (Thomas et al., 1997; Mitsiadis and Drouin, 2008).

Systems biology approaches indicated that Wnt and Bmp pathways might act as the two main mediators of epithelial-mesenchymal signalling in early tooth development (O'Connell et al., 2012). Bmp4 secretion together with Fgf4 induce the formation of the primary enamel knot (O'Connell et al., 2012; Jernvall et al., 1998; Järvinen et al., 2006). Through the secretion of a plethora of members of Wnt, Fgf, Bmp, and Shh signalling pathways the enamel knot regulates the growth and the folding of the dental epithelium, as well as the formation of the secondary enamel knots (Mitsiadis and Graf, 2009; Lan et al., 2014).

Notch signalling, a key factor in the process of cell fate decision, is also emerging as an important regulator of tooth development, in particular during ameloblast differentiation. In the developing teeth, Notch

receptors and their Jagged and Delta ligands are expressed in neighbouring cell layers of the dental epithelium (Mitsiadis et al., 1995a, 1997, 1998a; Mitsiadis and Graf, 2009). *Jagged2* mutant mice grow abnormally shaped teeth, which lack enamel (Mitsiadis et al., 2010).

Tooth innervation

The mature tooth is innervated by unmyelinated and myelinated sensory nerves, as well as unmyelinated sympathetic neurons. Sensory nerves originate from the trigeminal ganglion, while sympathetic adrenergic nerves grow from the superior cervical ganglion (Pagella et al., 2014a). Myelinated and unmyelinated axons intermingle and some of them project into the dentin through the dentinal tubules. Sensory fibres convey sensory information to the central nervous system, while sympathetic nerves regulate blood flow into the pulp and are also involved in the process of dentinogenesis (Johnsen, 1985).

At early developmental stages, fibres from the trigeminal nerve are located below the dental placode (Mohamed and Atkinson, 1983) (figure 4). At the bud and cap stages, nerve fibres abundantly innervate the dental follicle, while they are completely absent from the dental epithelium and the dental papilla (Mohamed and Atkinson, 1983). The first axons penetrate the dental pulp when dentin deposition starts. Following this first fibres, massive neuronal ingrowth leads then to the rich pulp innervation that is completed soon after tooth eruption (figure 4) (Mohamed and Atkinson, 1983). Different neurotrophins (NTs) and axon guidance molecules regulate tooth innervation. Neurotrophic factors such as NGF (Nerve Growth Factor), NT-3, NT-4, and BDNF (Brain-Derived Neurotrophic factor) and their receptors are already expressed in both dental epithelium and mesenchyme at early stages, before the bud stage (Mitsiadis and Luukko, 1995; Luukko et al., 1997; Pagella et al., 2014a). These molecules generally promote axonal growth. However, at this stage, nerve ingrowth towards the dental epithelium and the dental papilla is actively inhibited and regulated by semaphorins, and by semaphorin3a in particular(Moe et al., 2012; Kettunen et al., 2005). Semaphorin3a is expressed in the dental follicle and directs the growth of trigeminal axons along precise paths (Moe et al., 2012). Tooth innervation is thus actively inhibited throughout the early stages of development.

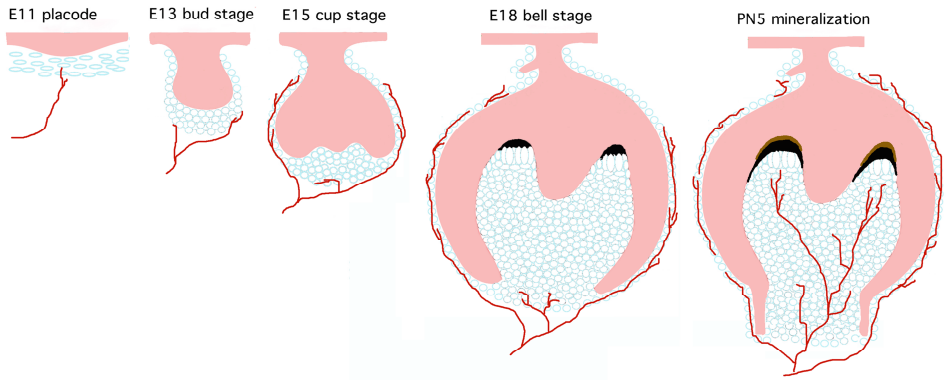


Figure 4. Schematic overview of tooth innervation at different developmental stages (Pagella et al., 2014a)

The role of innervation in tooth initiation and development is controversial. Organotypic *in vitro* and *ex vivo* cultures have suggested that tooth initiation and development can proceed without any neuronal contribution (Lumsend and Buchanan, 1986) since entire teeth have been developed in *ex vivo* cultures regardless of the presence or absence of trigeminal ganglia. In contrast, innervation is clearly necessary for tooth development in fishes. Denervation of the lower jaw in teleost fishes leads to the complete arrest of tooth formation and substitution, indicating that innervation is essential for tooth initiation (Tuisku and Hildebrand, 1994). Concerning mammalian tooth innervation, it has been demonstrated that sensory nerves regulate mesenchymal stem cell homeostasis in mouse incisors via secretion of Sonic Hedgehog (Shh). It is important to notice that the majority of the studies addressing the role of innervation in tooth development have focused on tooth initiation and morphogenesis. Surprisingly, no studies are available that would address the role of innervation in the later stages of tooth development and, in particular, in the formation of enamel and dentin.

The mouse tooth as a model for the study of development and regeneration

In each hemi-mandible of the mouse there are three molars that are separated from the incisor by a toothless region called diastema. Furthermore, mice have only one single set of teeth (figure 5A). Although humans have two sets of dentitions (lacteal and permanent) and two extra tooth types, the canines and premolars, their structure and development is highly similar to that of mouse teeth. Human teeth and mouse molars do not grow after eruption and, most importantly, can neither repair nor regenerate enamel upon tooth injury. However, mouse incisors are continuously growing teeth and thus constitute fundamental tools for studying developmental and regenerative events. Rodent incisors are asymmetrical, with enamel located only on their labial side (figure 5B). Their continuous growth ensures replenishment of enamel, which is lost due to their mastication habits. Enamel production is sustained by the continuous

generation of ameloblasts from the labial cervical loop (laCL), which is a niche hosting dental epithelial stem cells (DESCs) (figure 5B).

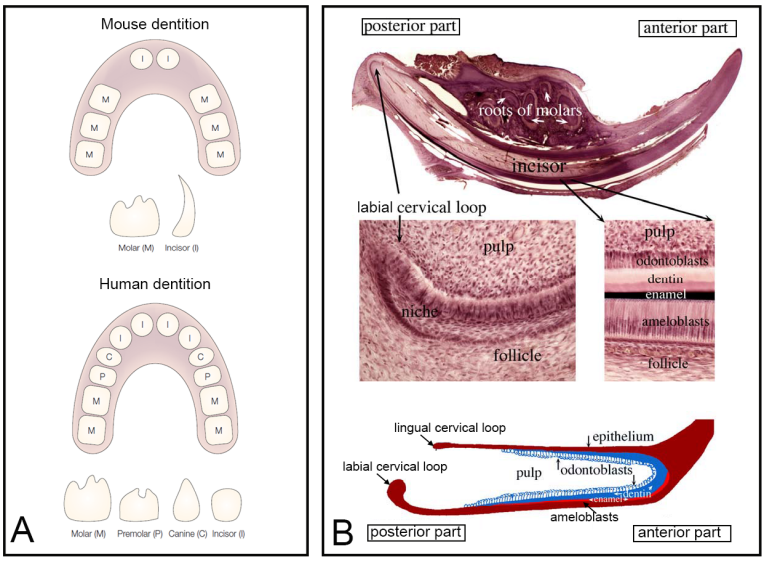


Figure 5. The mouse tooth as an experimental model. **A)** Comparison of mouse and human dentition – adapted from (Tucker and Sharpe, 2004)**; B)** Histological and schematic overview of the mouse incisor – adapted from (Bluteau et al., 2008)**.**

Therefore, mouse teeth represent extremely valuable models for studying the molecular bases of development and regeneration of organs. Mice with defective teeth can be used to study cellular, tissue and molecular alterations that could be lethal if other vital tissues or organs were affected. Furthermore, the continuously growing incisor allows to study the bases of tissue regeneration and to follow enamel development postnatally.

3.1 Nogo-A and Nogo proteins

Three Nogo proteins (Nogo-A, -B and -C) were discovered and extensively studied in the context of injury and repair of nerve fibres in the central nervous system (Schwab, 2010). Only recently, several studies shed light on the roles of the Nogo molecules during organ development and homeostasis. From these studies, Nogo proteins have emerged as key regulators of neuronal development, plasticity and growth. More generally, Nogo proteins are involved in cell motility and growth, as for example in neurons and blood vessels (Schmandke et al., 2014, 2013). Furthermore, Nogo proteins may have intracellular functions related to their localization in the endoplasmic reticulum (ER) (Schwab, 2010).

Nogo proteins: functional domains and structure

Nogo-A, -B, and -C are the three main products of the *Rtn4* gene, also known as *Nogo* (Schwab, 2010). (Schwab, 2010). The *Rtn4* gene is composed of 9 exons; alternative splicing or differential promoter usage gives rise to these three isoforms (Chen et al., 2000; GrandPré et al., 2000; Prinjha et al., 2000) (figure 6). All three isoforms share the same C-terminal reticulon homology domain (RHD), encoded by exons 4-9 and consisting of two hydrophobic membrane domains flanking a hydrophilic 66 amino acids long region, termed Nogo-66 (GrandPré et al., 2000). The N-termini on the contrary exhibit a large diversity (Schwab, 2010), indicating that different isoforms interact with different proteins and could thereby exert a wide variety of biological functions (Kempf and Schwab, 2013).

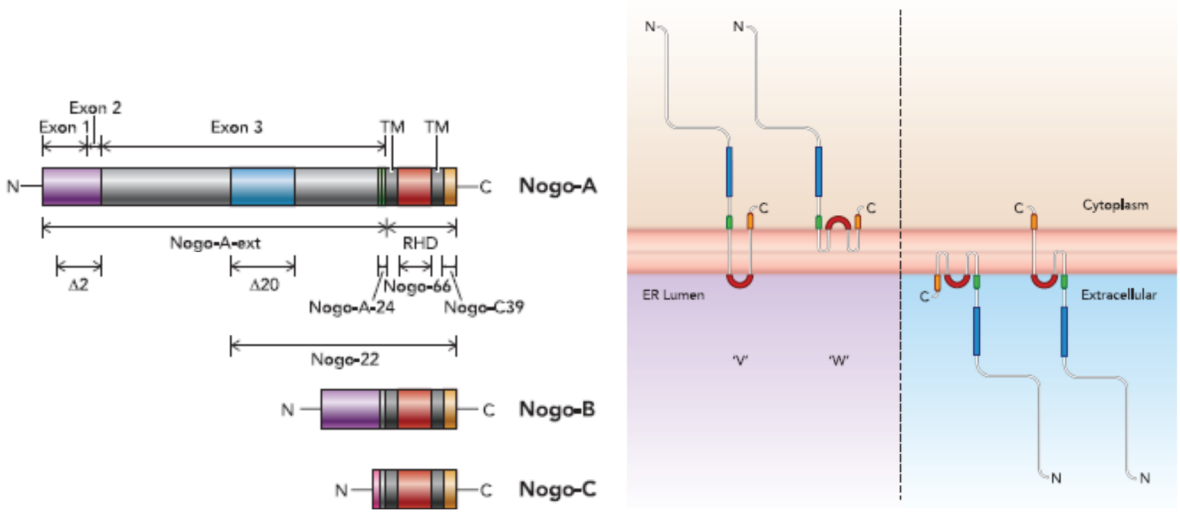


Figure 6. Structure and membrane topology of Nogo proteins. Left: schematic structure of the Nogo isoforms Nogo-A, Nogo-B and Nogo-C. All active Nogo-A domains are indicated (see following paragraphs). Right: multiple membrane topologies proposed for Nogo proteins at the ER membrane and at the plasma membrane**.** (Kempf and Schwab, 2013)

The N-termini of Nogo-A and Nogo-B are identical, consisting of a 172-amino acid sequence encoded by a single exon (exon 1), followed by a short region encoded by exon 2 and, in Nogo-A, by a 800-amino acids long domain encoded by exon 3 (figure 6) (Chen et al., 2000; Oertle et al., 2003b). This 800-aminoacid insert, as well as the N-terminus of Nogo-A and Nogo-B, contains large unstructured regions (Li and Song, 2007). No homologies of any of these exons with known protein sequences have been found so far. The shortest Nogo isoform, Nogo-C, has an N-terminus of just a few amino acids that is directly followed by the RTN domain. This N-terminus is encoded by a primary transcript that is generated from a different promoter to the one that generates the primary transcript for the N-termini of Nogo-A and Nogo-B (Oertle et al., 2003b).

Nogo-A is predominantly localized to the endoplasmic reticulum (ER) where it is required for the formation and maintenance of ER tubules *in vitro* (Voeltz et al., 2006). A small but significant amount of Nogo-A is localized on the cell surface of oligodendrocytes, neurons, and some non-neuronal cell types (Dodd et al., 2005; Oertle et al., 2003a).

Nogo proteins can have different membrane topologies, in particular in regard to the extracellular versus cytoplasmic localization of the N-terminus and the exon 3-encoded domain. On the cell surface, the N termini of Nogo-A and Nogo-B, and the exon 3 sequence of Nogo-A have been found to face the extracellular space, even though they do not contain a conventional signal peptide for ER translocation. However, the N-terminus of Nogo-A and Nogo-B can also face the cytosol, in particular when Nogo is located in the ER (Schwab, 2010; Dodd et al., 2005; Oertle et al., 2003a).

Nogo-A: receptors, interaction partners and signalling

Nogo-A interacts with various receptors via different domains and sequences: Nogo-66, Nogo-Δ20 (aminoacids 544-725 of rat Nogo-A) and the N-terminal domain (see figure 6, 7).

Receptors for Nogo-66.

The first Nogo receptor to be characterized was the glycosylphosphatidylinositol (GPI)-linked leucine rich repeat (LRR) protein Nogo receptor 1, known as NgR1 (Schwab, 2010). NgR1 binds mainly to the Nogo-66 (figure 6, 7) loop, but a sequence at the C-terminal of the Nogo-A-specific region encoded by exon 3 increases the affinity of Nogo-A to NgR1 (GrandPré et al., 2000; Schmandke et al., 2014). NgR1 has no transmembrane domain, and therefore depends on co-receptors to transduce signals. It was shown that NgR1 associates and interacts with the low affinity neurotrophins receptor p75 (P75-NGFR), its relative tumor necrosis factor-α (TNF-α) receptor superfamily member 19 (TROY), and LINGO1 (Wang et al., 2002; Spencer et al., 2003; Yiu and He, 2006; Shao et al., 2005; Park et al., 2005). NgR1 does not interact only with Nogo-A, but it was shown to bind also integrins, myelin-associated glycoprotein (MAG), and

oligodendrocyte myelin glycoprotein (Wang et al., 2002; Domeniconi et al., 2002). In addition to NgR1, Nogo-66 interacts also with the paired immunoglobulin-like receptor B (Atwal et al., 2008).

Nogo-A specific receptors.

The extracellular region of Nogo-A, including the N-terminal, the exon3 encoded domain, and in particular the region termed Nogo-A-Δ20 (figure 6, 7) exert the strongest effects on axonal growth and, unlike Nogo-66, they also influence cell migration of non-neuronal cells *in vitro* (Chen et al., 2000; GrandPré et al., 2000; Oertle et al., 2003a). Nevertheless, the specific receptor for Nogo-A remained elusive for long time. Only recently the G protein-coupled receptor (GPCR) sphingosine 1-phosphate receptor 2 (S1PR2) was identified as the first functional receptor for the inhibitory, Nogo-A-specific, Nogo-A-Δ20 domain (Kempf et al., 2014).

S1PR2 belongs to a subfamily of five S1PRs (Spiegel and Milstien, 2003). S1PRs are generally activated by the low molecular weight lipid ligand sphingosine 1-phosphate (S1P), and mediate diverse receptor-specific effects on various cell types, including cytoskeletal dynamics, cell motility and apoptosis (Spiegel and Milstien, 2003). In the nervous system, activation of S1PR2 leads to neurite retraction, consistently with the effects of Nogo-A-Δ20 (Kempf et al., 2014). S1PR2 is expressed on a wide variety of cell types. The cell-specific interplay of S1PR2 with known receptors and co-receptors for Nogo-A needs to be investigated in detail with regard to their corresponding physiological effects. In this light, it was proposed that the Δ20 domain of Nogo-A binds to S1PR2 and the Nogo-66 loop to NgR1, resulting in the formation of a multi-site/multi-ligand receptor complex (Kempf et al., 2014). The inhibitory effects of this complex might potentially be further amplified by additional Nogo-A co-receptors and downstream effectors (Kempf et al., 2014).

Second messengers and downstream signalling pathways

The different active regions of Nogo-A can trigger the activation of the small GTPase RhoA and its effector protein ROCK (Rho-associated coiled coil containing protein kinase 1) in different neuronal subtypes (Schwab, 2010; Yiu and He, 2006; Shao et al., 2005; Park et al., 2005). Rho GTPases, including RhoA, Rac1 and Cdc42, integrate several molecular inputs and trigger downstream cytoskeletal rearrangements, such as actin polymerization for the growth of lamellipodia and filopodia or actin depolymerisation for cell or axonal retraction (Schmandke et al., 2007). The main downstream effectors of the Nogo-A/RhoA/ROCK axis include myosin light chain (MLC II) and cofilin, which promote myosin II contractile activity or actin depolymerisation, respectively. Besides affecting the actin cytoskeleton, Nogo-66 also destabilises microtubule assembly through phosphorylation of the collapsin response mediator protein 2 (CRMP2). In addition, Nogo signalling results in the inactivation of glycogen synthase kinase 3β (GSK3β), which leads to stabilized RhoA-CMPR4 dependent inhibition of axonal growth (figure 7) (Alabed et al., 2010).

Apart from the well-known targeting of RhoA-ROCK signalling pathway, little is known for Nogo-A in terms of intracellular signalling cascades. First evidence was provided for transcriptional changes, induced by Nogo-A-Δ20 (Joset et al., 2010; Schmandke et al., 2014). Even though it was observed that Nogo-A-Δ20 induces a reduction in the levels of phosphorylated cyclic AMP response element binding (pCREB) (Joset et al., 2010), not much is known about direct transcriptional changes induced by Nogo-A in neurons. RNA sequencing analysis of spinal cord tissue from Nogo-A KO versus wild type mice showed regulation of genes related to cytoskeleton, transport and signalling (Kempf et al., 2013). The cellular and molecular bases of these changes and the role that transcriptional mechanisms play in these regulations remain to be determined.

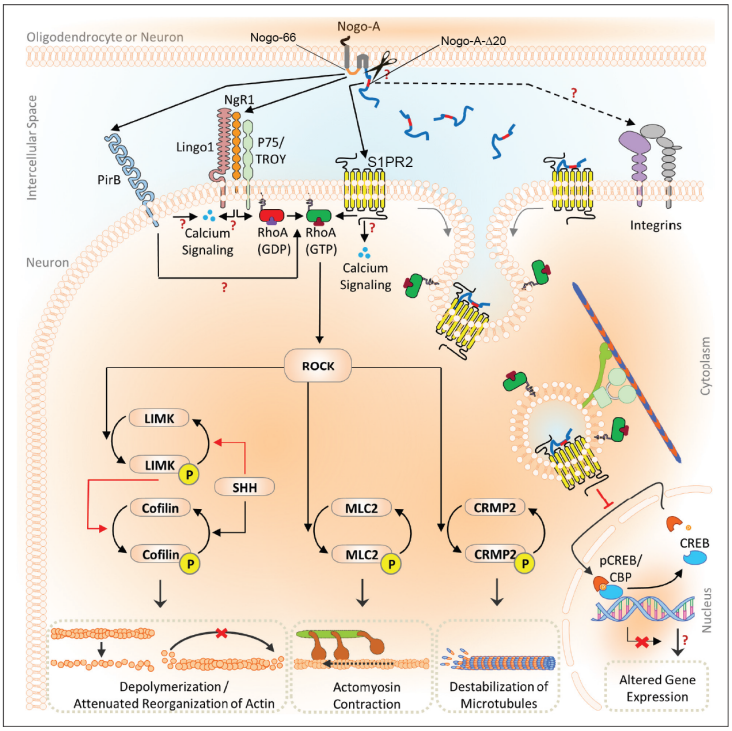


Figure 7. Main targets of Nogo-A signalling, adapted from (Schmandke et al., 2014).

Nogo-A functions and roles

Nogo-A roles in the development of the nervous system

During development Nogo-A is mainly expressed in both peripheral and central neurons (PNS and CNS, respectively) (Huber et al., 2002). During this process Nogo-A regulates several aspects of neural development and differentiation.

Several experiments showed that Nogo-A can regulate the migration of a plethora of different cell types in the CNS, including neural stem cells, neuroblasts and microglia (Rolando et al., 2012; Mathis et al., 2010;

Mingorance-Le Meur et al., 2007; Su et al., 2007). Interestingly, the effects of Nogo-A on cell spreading and migration can vary completely depending on the developmental stage and on the cell type.

During neuronal cytodifferentiation Nogo-A is highly expressed in outgrowing axons *in vivo* (Tozaki et al., 2002; Hunt et al., 2003; Mingorance-Le Meur et al., 2007). However, its action on growing axons during development is not univocal. For example, embryonic cortical neurons lacking Nogo-A, Nogo-B and Nogo-C show increased branching in culture (Mingorance-Le Meur et al., 2007). On the contrary, dorsal root ganglion explants from new-born mice or rats treated with antibodies against Nogo-A, NgR1 or LINGO1 , or isolated from Nogo-A null mice, show a highly fasciculated outgrowth with reduced branching and longer neurites (Petrinovic et al., 2010; Schwab, 2010). These results suggest that Nogo-A can regulate axonal adhesion and branching in developing nerve fibres. At the same time, it is possible that the output of Nogo-A-dependent signalling in these processes might strongly depend on the developmental stage and/or the origin of the neurons involved.

Nogo-A is also involved in axon guidance. For example, during mouse embryonic development both optic nerve axons and spinal commissural axons are repulsed by Nogo-A expressing glia in the ventral midline. Blocking Nogo-A function via a blocking antibody or using a peptide blocking NgR1 leads to the maturation of misprojecting axons (Schwab, 2010; Wang et al., 2008a, 2008b). At early postnatal stages, the precise channelling of sensory tracts of the spinal cord to their target territories requires Nogo-A. Nogo-A deletion or the ablation of Nogo-A expressing oligodendrocytes lead to an aberrant and spread distribution of corticospinal fibres (Schwab and Schnell, 1991; Schwab, 2010).

Nogo-A could also be involved in the formation of myelin. At late developmental stages, both axons and the surrounding myelin-forming oligodendrocyte express Nogo-A and members of the class of Nogo receptors. It was shown that mice lacking both Nogo-A and MAG show delays in myelin formation and have defective myelin (Pernet et al., 2008).

Taken together these results indicate that Nogo-A is a major regulator of neural development, being involved in cell migration, axonal outgrowth and axon guidance during the whole period of the development of the nervous system.

Nogo-A roles in the adult CNS

Little is known concerning the role of Nogo-A in the adult CNS. Nogo-A was shown to be involved in the establishment of mature connections and the subsequent loss of plasticity in different districts of the CNS. For example, in the visual cortex, adult mice lacking both Nogo-A and Nogo-B, or mutant mice lacking NgR1 or PIRB, showed significantly increased ocular dominance plasticity, which was comparable to those in immature mice (McGee et al., 2005; Syken et al., 2006).

Within the sub-ventricular zone (SVZ) Nogo-A has a dual role in the regulation of neuroblasts differentiation and migration. The Nogo-66/NgR1 axis inhibits neural stem cell proliferation, while at the

same time, the Nogo-A-Δ20 domain promotes neuroblasts migration towards the olfactory bulb (Rolando et al., 2012). Thus, Nogo-A regulates the pace of neurogenesis in the adult SVZ and the migration of neuroblasts towards the olfactory bulb (Rolando et al., 2012)

Increasing evidence suggests that Nogo-A is involved in the regulation of synaptic plasticity, both in long term potentiation (LTP) and long term depression (LTD) (Delekate et al., 2011; Raiker et al., 2010; Lee et al., 2008). There are also indications that Nogo-A might be implicated in psychiatric disorders such as schizophrenia. There is in fact an interesting correspondence between schizophrenia-like behaviours in Nogo-A knockout mice and the genetic associations between psychiatric disorders and NgR/Nogo mutations in certain families and patients (Hsu et al., 2007; Willi et al., 2010; Sinibaldi et al., 2004; Tan et al., 2005; Novak et al., 2002). This association could be due to deficits in Nogo signalling during development, but also due to alterations in synaptic and wiring stability in the adult CNS.

Intracellular functions of Nogo proteins

Relatively few studies addressed the intracellular functions of Nogo-A. Based on studies of molecular interactions, it has been suggested that Nogo proteins can bind and inhibit the enzyme β-secretase (BACE) in the adult CNS, thus inhibiting the production of β-amyloid peptides, which are linked to Alzheimer's disease (He et al., 2004; Murayama et al., 2006). There is however no direct evidence for a crucial role of Nogo-A or Nogo-B in the pathogenesis of Alzheimer's disease. In different studies it was observed that Nogo-A is upregulated in conditions of cellular stress and intracellular Nogo-A antagonizes reactive oxygen species formation in primary immature neurons *in vitro*, protecting them from oxidative stress (Gil et al., 2006; Mi et al., 2012). Another intracellular interaction partner for Nogo proteins and the RTN3 is the anti-apoptotic protein BCL2, thus suggesting a possible link between Nogo proteins and apoptotic events (Tagami et al., 2000; Schwab, 2010; Sutendra et al., 2011).

Nogo proteins, together with the other RTN proteins, are highly present in the ER, thus suggesting additional roles related to this localization (Schwab, 2010; Teng and Tang, 2008)

Nogo-A in CNS injury and regeneration

The vast majority of the studies involving Nogo-A were conducted in the context of CNS injury and repair. Nogo-A was identified in the late 80s as one of the two membrane protein fractions from CNS myelin inhibiting neurite outgrowth *in vitro* (Caroni and Schwab, 1988b). The antibody-mediated neutralization of these fractions was shown to largely eliminate their neurite-outgrowth inhibition properties and to allow extensive axonal regeneration and functional recovery *in vivo* (Caroni and Schwab, 1988a). Since then, several different approaches, including blocking antibodies and peptides (anti-Nogo-A and anti-NgR1) and mutant animals (Nogo-A null mice, NgR1-null mice, Nogo-A knockdown rats, ROCKII null mice) have been

used extensively to investigate the impact of Nogo-A on CNS injury (Zörner and Schwab, 2010; Simonen et al., 2003; Duffy et al., 2009; GrandPré et al., 2002; Kim et al., 2004; Li et al., 2004).

In spinal cord injuries, treatment of injured rodents and primates with inhibitors of Nogo-A, the Nogo-receptor complex or the downstream Rho/ROCK pathway enhanced regenerative and compensatory sprouting. Treated animals show significantly higher functional recovery than control and non-treated animals. Moreover, negative side effects were absent. On the basis of these observations, a phase I clinical trial with intrathecal infusion of a Nogo-A blocking antibody was conducted onto patients with acute and severe spinal cord injury confirming the absence of negative side effects also in humans (Silver et al., 2014).

Similarly, in stroke models, the blocking of Nogo-A or NgR1 increased compensatory sprouting of uninjured nerve fibres (Cafferty et al., 2010; Lindau et al., 2014). It was demonstrated that the anti-Nogo-A therapy is fundamental in the recovery of lost motor function and the pattern of fibre sprouting after large forebrain cortex strokes (Wahl et al., 2014). Nogo-A is also under intense study in several neural pathologies, including amyotrophic lateral sclerosis, Alzheimer's disease and multiple sclerosis (Schmandke et al., 2014).

Nogo-A roles outside the CNS

Although Nogo-A is expressed in several tissues outside the CNS, a surprisingly limited amount of studies investigated the role of Nogo-A outside neural development and physiology. Different studies investigated the roles of Nogo-B in vascular remodelling and functionality (Acevedo et al., 2004) and in the immune system (Di Lorenzo et al., 2011; Schanda et al., 2011). However, only recently it has been shown that Nogo-A acts as an inhibitor of CNS angiogenesis. Interestingly, the mechanisms involved in this process are similar to those that mediate Nogo-A-dependent inhibition of axonal growth. In fact, in the endothelial cells the Nogo-A-Δ20 region signals by activating the ROCK/RhoA cascade that inhibits their spreading, adhesion and migration (Wälchli et al., 2013). As a consequence, Nogo-A deletion or its antibody-mediated neutralization lead to hypervascularization of the CNS (Wälchli et al., 2013).

Another single study investigated the role of Nogo-A in bone homeostasis (Lee et al., 2012). In particular, it was shown that a knockdown of Nogo-A significantly reduced RANKL-dependent formation of mature osteoclasts from bone marrow macrophages (BMMs). Conversely, Nogo-A overexpression in BMMs led to increased osteoclastogenesis (Lee et al., 2012). I*n vivo*, Nogo-A knockdown in the mouse calvariae could prevent interleukin 1 (IL-1) induced bone loss.

However, apart from these two single studies, no information is available about the possible functions of Nogo-A outside the CNS.

4. Aims of the project

The orofacial complex is richly innervated, and this dense network of nerve fibres regulates key functions and the homeostasis of the different organs (Pagella et al., 2014a). Nevertheless, neither the mechanisms regulating orofacial innervation nor the effects of innervation on orofacial organs, and in particular teeth, are known. As a potent regulator of axonal growth and regeneration in the CNS, Nogo-A might regulate orofacial innervation as well and, as a result, the physiology of the various tissues in this region. Nogo-A might also play a role in the development, homeostasis and regeneration of teeth that is not strictly linked to their innervation, as the results obtained on bone suggest.

Therefore, the project follows four principal aims:

1) Description of Nogo-A expression in the orofacial complex, and more precisely in teeth. No data regarding the expression of Nogo-A in the orofacial complex is currently available. Therefore, a precise description of Nogo-A expression at different developmental stages is needed in order to start any investigation concerning its role in the orofacial compartment and teeth.
2) Study of Nogo-A functions on tooth development. We investigate the *in vivo* effects of Nogo-A deletion in the teeth of a mouse Nogo-A KO model (Simonen et al., 2003).
3) Identification of the mechanisms associated with Nogo-A functions in the orofacial complex. We use molecular analysis in order to understand which molecular mechanisms are affected by the deletion of Nogo-A. In parallel, we perform *in vitro* studies aimed to dissect the precise function of Nogo-A in tooth innervation and development.
4) Nogo-A in regenerative processes. Using a model of tooth injury in mice, we investigate whether Nogo-A may be involved in tooth regeneration.

5. Materials and methods

Tissue specimens and processing

Mice

Animal housing and experimentation were performed according to the Swiss Animal Welfare Law and in compliance with the regulations of the Cantonal Veterinary Office, Zurich.

Wild type C57/BL6J were used at different embryonic and postnatal stages. Nogo-A KO (-/-) mice were previously generated and kindly provided by the group of Prof. Martin Schwab (Simonen et al., 2003) (Institute for Brain Research, UZH and ETH Zurich). The day when a vaginal plug was detected was counted as embryonic day (E)0, and the day of birth was considered as postnatal day (P)0. The stages of embryonic development were confirmed according to morphological criteria.

Mouse genotyping was performed via PCR using the following primers:

- Fw: 5' TGC TTT GAA TTA TTC CAA GTA GTC C 3'
- Rv1: 5' CCT ACC CGG TAG AAT ATC GAT AAG C 3'
- Rv2: 5' AGT GAG TAC CCA GCT GCA C 3'.

Tissue collection

Embryonic tissues for histological and immunohistological analyses were dissected in cold PBS and fixed overnight in 4% paraformaldehyde (PFA). Pups and adult mice were perfused intracardially with 4% PFA, followed by a post-fixation of the head was then post-fixed in 4% PFA for 2-7 days.

For histology, tissues were decalcified for 10 weeks in 10% ethylenediaminetetraacetic acid (EDTA), dehydrated, embedded in paraffin and sectioned at 5-10 µm for *in situ* hybridization, immunohistochemistry and hematoxylin/eosin staining. Decalcification was not performed on embryonic tissues.

For organ cultures (trigeminal ganglia, teeth) and whole mount immunofluorescence embryos were decapitated and teeth and trigeminal ganglia dissected in cold Dulbecco's phosphate buffered saline (PBS).

Histology

Hematoxylin/eosin stanings were performed on paraffin sections. Rehydrated sections were incubated for 1' in filtered hematoxylin, washed with tap and bi-distilled H_2O, incubated for 1' in eosin, rehydrated and mounted with Eukitt.

In situ hybridization, immunohistochemistry and immunofluorescence

In situ hybridization

In situ hybridisation was performed on paraffin sections or cryosections as previously described (Mitsiadis et al., 1995b). 400 bp fragment of mouse cDNA were subcloned into a pBlueScriptII SK plasmid. Digoxigenin-UTP-labeled single-stranded antisense RNA probes were prepared by standard procedures. The plasmid vectors were linearized with EcoRI and/or HindIII restriction enzymes according to the probe insertion orientation (Promega). The labelled probes were ethanol-precipitated, resuspended in 100 mM DTT, diluted in hybridization solution (60% deionized formamide, 20 mM Tris-HCl, 5 mM EDTA, pH 8, 0.3 M NaCl , 0.5 mg/ml yeast RNA, 5% dextran sulfate). In situ hybridization was performed via incubation with the probe at 60°C. After intense washing, the slides were incubated in blocking solution (MABT + 20% Normal Goat Serum) and incubated with Anti-digoxigenin (DIG)-AP (alkaline phosphatase conjugate) Fab fragment (Boehringer Mannheim, 1093 274) diluted 1:1000 in blocking solution. The colour reaction was developed using Nitro Blue Tetrazolium (NBT, Sigma N-6876) and 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP, Sigma B-8503) in staining solution 2% NaCl, 5% $MgCl_2$, 10% Tris-HCl pH 9.5, 1% Tween-20 and 0.5% Levamisole.

Antibodies

The following primary antibodies were used: mAb anti-Nogo-A 11C7 (1µg/ml, (Oertle et al., 2003a)), rabbit AS “Laura” anti-Nogo-A (1:500-1:8000, (Oertle et al., 2003a), mouse IgG1 anti-neurofilament (2H3, 1:50-1:100, Hybridoma Bank, Iowa City, USA), rabbit AS anti-Notch1 (1:800, (Mitsiadis et al., 1995a)), rabbit AS anti-Notch2 (1:800, (Mitsiadis et al., 1995a)), rabbit IgG anti-peripherin (1:1000, ab4666, Abcam, Cambridge, UK), goat IgG anti-NgR1 (AF1440, R&D Systems, Minneapolis, USA), human IgG anti-S1PR2 (1:300, (Kempf et al., 2014)), rabbit IgG anti-amelogenin (1:100, ab153915, Abcam, Cambridge, UK), IgG anti-DSPP (1:200, ab122321, Abcam, Cambridge, UK), rat IgG2a anti-BrdU (1:50-1:100, BU1/75,BioRad-AbD Serotec, Oxford, UK).

For immunofluorescence, the following secondary antibodies were used: goat anti-rabbit Alexa 488- and Alexa 568-conjugated, goat anti-mouse IgG Alexa 488- and 568-conjugated, goat anti-rat IgG Alexa 568-conjugated (Life Technologies Europe, Switzerland).

Immunohistochemistry

Immunohistochemistry was performed on 5-10 µm thick paraffin sections or cryosections. Cryosections were fixed with cold acetone onto the slides; paraffin sections were rehydrated before staining. Endogenous peroxidases were quenched by incubating sections in 3-6% H_2O_2 in ice-cold methanol for 30’. For specific stainings, heat-induced antigen retrieval in 10mM trisodium citrate buffer, pH 6.0, was performed. For the staining procedure, the Vectastain ABC Kit was used (ABC Kit, Vector Laboratories,

Servion, Switzerland). As chromogenic substrate, SIGMAFAST™ 3,3'-Diaminobenzidine tablets (Sigma-Aldrich, D4293) were used. Omission of the primary antibody served as negative control. Stained sections were counterstained with toluidine blue or hematoxylin (both diluted 1:10 in distilled H_2O) and mounted with Glycergel (C0563, Dako, Dako North America Inc.) or Eukitt® (03989, Fluka, Sigma-Aldrich Chemie, Germany). Pictures were taken using the Leica DFC420C camera and the Leica Application Suite (LAS) software.

Whole mount immunofluorescence

For anti-BrdU whole mount immunofluorescence, cultured samples were fixed in paraformaldehyde 1% for 15'. Samples were then incubated in 2N HCl at 37°C for 30'. Blocking was performed overnight at 4°C in blocking buffer (PBS + 10% normal goat serum (NGS) + 0,2% Triton-X100 + 2% Bovine Serum Albumin (BSA)). Samples were then incubated with primary antibodies (diluted in blocking buffer) for 48-72 hours. Primary antibodies were detected incubating for 48-72 hours the samples in fluorescent secondary antibodies (see paragraph above) diluted in blocking buffer. All samples were also stained with DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich Chemie, Germany). Finally, samples were cleared by incubating them for 24 hours in FocusClear© (CelExplorer Labs Co.) and imaged via confocal fluorescent microscopy (CLSM Leica SP5 Mid UV-VIS). Images were analysed using Imaris 7.7.2 (Bitplane AG, Oxford Instruments, Switzerland).

Micro-computed tomography (µCT)

Adult perfused mouse heads were washed in PBS and progressively dehydrated to 70% ethanol for µCT analysis. The µCT scans were performed using a commercially available µCT unit (Specimen µCT 40, Scanco Medical, Brüttisellen, Switzerland) with all imaging parameters kept identical during all examinations (tube voltage, 70 kV, tube current 114 µA; isotropic resolution, 18 µm). The original images were converted into the RAW-format using the proprietary software of the µCT device and imported in the 3D reconstruction program VGStudio Max (Volume Graphics, Heidelberg, Germany). All the analysed samples were segmented manually using wild type teeth as reference for the grey level values corresponding to the single mineralized tissues (enamel, dentin, bone).

Backscattered scanning electron microscopy (SEM) analysis

Fully mineralized lower hemi-jaws were dissected from perfused adult (approx. 3 months of age) wild type and Nogo-A KO mice. Soft tissues were removed manually and by incubating the samples in H_2O_2 3% overnight. The lower jaws were then dehydrated and embedded in Technovit 7200 VLC (Heraeus Kulzer, Wehrheim, Germany). Light-polymerized blocks were mounted on aluminium stubs, polished and coated

with a 10-15 nm thick layer of carbon. Thereafter, they were examined using a Tescan EGATS5316 XMSEM (Tescan, Brno, Czech Republic) operated in BSE mode. Elemental composition of enamel was analysed with the aid of energy-dispersive X-ray spectroscopy (EDS). A Si(Li) detector (Oxford Instruments, Wiesbaden, Germany) served for recording EDS spectra using an accelerating voltage of 7 kV, a working distance of 23 mm, and a counting time of 100 s. For the quantitative analysis of these spectra, the Inca energy software (Oxford Instruments) was used.

Transmission electron microscopy (TEM) Analysis

Lower hemi-jaws were dissected from perfused adult (approx. 3 months of age) wild type and Nogo-A KO mice and decalcified in 10% EDTA (pH 7.4) for 10 weeks. The samples were then postfixed in 1.33% Os-tetraoxide in 0.067 M s-collidine buffer for 2 hours at room temperature. Thereafter, they were dehydrated in ethanol, transferred to propylene oxide and embedded in Epon 812 (Fluka, Buchs, Switzerland). From the resin blocks, thin sections of 80-100 nm in thickness were cut using a Reichert Ultracut ultramicrotome (Leica Microsystems, Heerbrugg, Switzerland) and diamond knives (Diatome, Biel, Switzerland). Sections were collected on copper grids, contrasted with U-acetate and Pb-citrate, and examined in a Philips EM400 T TEM (FEI, Eindhoven, Netherlands) at 60 kV. Micrographs were recorded using a Hamamatsu ORCA-HR camera (Hamamatsu Photonics, Hamamatsu, Japan) and the AMT image acquisition software (Deben, Bury St. Edmunds, UK).

RNA sequencing and real time PCR

Samples were isolated from lower incisors freshly dissected from newborn (p0) wild type and Nogo-A KO pups. RNA was isolated using the RNeasy Plus Mini Kit (Qiagen AG, Switzerland) and subsequently purified by ethanol precipitation.

Reads were quality-checked with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Low-quality ends were clipped (5 bases from the start, 3 bases from the end). Trimmed reads were aligned to the reference genome and transcriptome (FASTA and GTF files, respectively, downloaded from the Ensembl GRCm38) with STAR, version 2.3.0e_r291 (Dobin et al., 2013) with default settings.

The distribution of the reads across the transcriptome was recovered using the R package GenomicRanges (Lawrence et al., 2013) from Bioconductor version 3.0. The same package was used to quantify transcripts at the gene level. Isoform-specific quantification was calculated with RSEM, Version 1.2.21 (Li and Dewey, 2011). Differentially expressed genes were identified using the R packages edgeR (Robinson et al., 2010) from Bioconductor, version 3.0, with default settings. Gene sets were extracted for each comparison by selecting those genes with a log(fold-change) larger, in absolute value, than 0.5 and a

P-value smaller than 0.05. Based on these gene sets, the subsequent pathway analyses were performed with Metacore from Thomson Reuters. All clustering plots (heatmaps) have been produced using the package heatmap.2 from Bioconductor Version 3.0. Unsupervised clustering is based on the inclusion of all the genes that passed the quality filters and have been reported as expressed by the package GenomicRanges (Lawrence et al., 2013).

RNA sequencing results were validated via real time PCR. RNA retrotranscription was performed using the iScript™ Reverse Transcription Kit (Bio-Rad, Switzerland). Real-time PCRs were performed in an Illumina Eco real-time PCR System (Illumina), using Power Sybr Green ® (Life Technologies, Switzerland) as reaction mix.

The following primers were used:

Gene	Common name	Primer Forward 5'-3'	Primer Reverse 5'-3'
Hba-a	Hemoglobin alpha	CTA GCT TCC CCA CCA CCA AG	GGA GCT TGA AGT TGA CGG GA
Hbb-b	Hemoglobin beta	AGG TGA ACG CCG ATG AAG TT	AGC TTG TCA CAG TGG AGC TC
vWF	Von Willebrand Factor	GGC GTT ATA ACA GCT GTG CG	GAC AGT GTG CAG GGT CAT CA
Lyve1	Lymphatic vessel endothelial hyaluronic acid receptor 1	CAA CGA GGC CTG TAA GAT GC	AGG AGT TAA CCC AGG TGT CG
Uhrf1	Ubiquitin-like, containing PHD and RING finger domains, 1	TGT GGT CCG GAA CAT GAA GG	CCC TCT CTC TGG CCA GTA CT
Acta1	Actinin, alpha 1	TGC GCG ACA TCA AAG AGA AG	TGT CGC ACT TCA TGA TGC TG
Actb	Actin, beta	CCT CTA TGC CAA CAC AGT GC	CCT GCT TGC TGA TCC ACA TC
Ttn	Titin	GGG TCC GAG CTG AAA ACA GA	GTC CCA CGC GAC AAA TAT GC
Myot	Myotilin	CAC CGT GTC TTC CTC TGC TT	TGG AGT GGG CCT AGC ATT TG
Myh8	Myosin, heavy chain, 8	ACG GAG AGG AGC AGG AAG AT	TCC ACT TCA CTC TGC AGC TG
Lgals7	Galectin 7	TTT AAC CCG AGG CTG GAC AC	GCC TTG AAG CCT TCC TCT GT
Lgals8	Galectin 8	CAG ATC GAC ACA GTG GGC AT	CTT CAA ATG GCA GGC TCA GC
Mt1	Metallothionein 1	TCC TGC AAG AAG AGC TGC TG	CTT TGC AGA CAC AGC CCT GG
Mt2	Metallothionein 2	GCA AGA AAA GCT GCT GCT CC	CTT GTC GGA AGC CTC TTT GC
s1pr2	Sphingosine 1-phospate receptor 2 -S1PR2	GGA GAC TTT GGG CTC TCA CT	CCA ACC TGG GCT ACA TGA GA
Rtn4r	Nogo Receptor 1 -NgR1	CAG ACC GTG ATC TTA AGC GC	GGG TTC CAG TAC TGA GGC TT
Rtn4A	Nogo-A	CAG TGG ATG AGA CCC TTT TTG	GCT GCT CCT TCA AAT CCA TAA
Rtn4B	Nogo-B	GGC TCA GTG GTT GTT GAC CT	GGC CTT CAT CTG ATT TCT GG

Microfluidic co-cultures

Trigeminal ganglia were dissected from E15.5–E16.5 wild type and Nogo-A KO mouse embryos. Tooth germs were dissected from E16.5 wild type and Nogo-A KO embryos and from P5 wild type pups. Dissections were performed in cold PBS. Trigeminal ganglia were cultured in a Neurobasal medium (Gibco) supplemented with B27 (Gibco 17504-044), L-glutamine, 20 U/ml penicillin/streptomycin and 50 ng/ml NGF. Molars were cultured in a medium containing DMEM (high glucose 4.5mg/ml) (GE Healthcare, UK), 20% Fetal Bovine Serum (FBS) (Pansera, Germany), L-Glutamine, 20 U/ml penicillin/streptomycin and 0.9mM ascorbic acid (Pagella et al., 2015a, 2014b)

Microfluidic devices (AX150, AX450, Millipore, Switzerland) were punched with a 1mm diameter biopsy punch. Devices were assembled and coated with 0.1mg/ml poly-D-lysine and 5µg/ml laminin as previously described (Pagella et al., 2014b). Trigeminal ganglia were placed in the co-culture platform immediately

after dissection. Co-cultures were performed at 37◦C in a 5% CO2 incubator. Co-cultures were maintained for 10 days and media were changed every 72 hours. After culture, samples were washed with PBS and fixed with 4% PFA for 15'.

Tooth organ cultures

Tooth germs were carefully dissected from the lower jaws of new-born (p0) C547/BL6 mice as previously described. Tooth germs were then placed on top of a 0.1-0.8 µm Whitman filter on a stainless steel wire mesh (0.25 mm diameter wire) in an organ culture dish containing medium composed of DMEM (high glucose 4.5mg/ml) (GE Healthcare, UK), 20% Fetal Bovine Serum (FBS) (Pansera, Germany), L-Glutamine, 20 U/ml penicillin/streptomycin, and 0.9 mM ascorbic acid. Tooth germs were cultured in a humidified atmosphere of 5% CO_2.

24 hours after dissection, cultured tooth germs were incubated with 5-bromo-2'-deoxyuridine (BrDU, 40µg/ml) for 30'. Tooth germs were then treated with the Nogo-A blocking antibody 11C7 ((Oertle et al., 2003a), 30µg/ml) dissolved in the culture medium; samples cultured in absence of 11C7 served as controls. Culture medium was changed every 48 hours.

Tooth germs were fixed in 1% PFA for 15' and then processed for whole mount immunofluorescence or cryosectioning.

Tooth injury

Tooth injury was performed on adult Nogo-A KO mice and wild type littermates. One lower incisor was cut without damaging the inner dental pulp. The health status of the mice was assessed daily. Regrowth of the incisors was documented by conventional photography during mouse restraining.

6. Results

Nogo-A expression during development

The expression of Nogo-A outside the central nervous system is nearly unknown; in particular, no data is available regarding the expression of Nogo-A in the orofacial complex during development.

We analysed the expression of Nogo-A in the orofacial complex via *in situ* hybridization at birth (P0-P1). At this stage *Nogo-A* is clearly expressed in several orofacial tissues, such as molars (figure 8A, B), trigeminal ganglia (figure 8C), the frontonasal region (figure 8D), hair follicles (figure 8E), and lacrimal (figure 8F) and salivary glands.

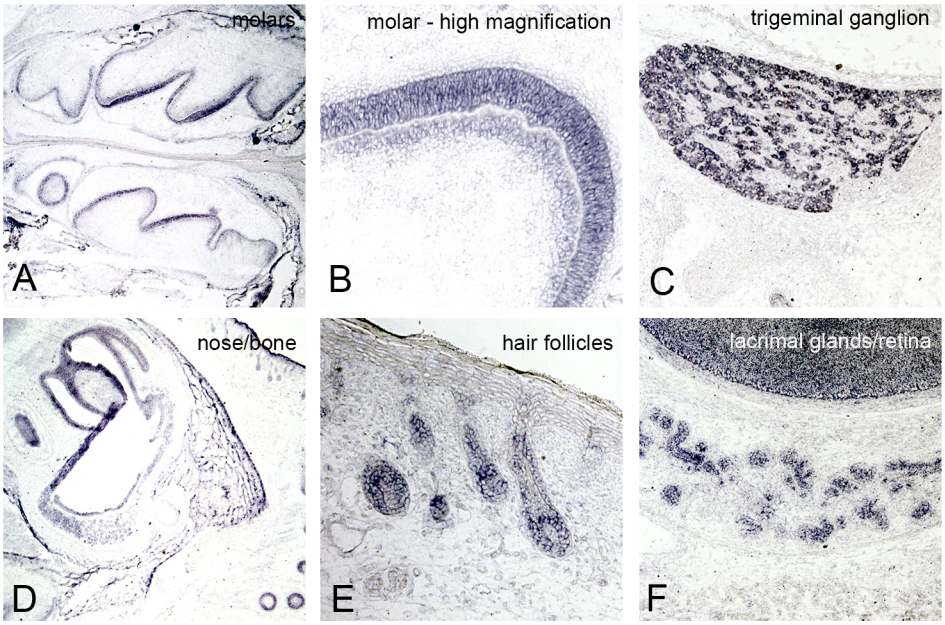


Figure 8. *In situ* hybridization showing Nogo-A mRNA expression at birth. **A)** molars; **B)** molars- higher magnification; **C)** trigeminal ganglion ; **D)** nose/bone; **E)** hair follicles; **F)** lacrimal gland/retina.

Based on the results from the *in situ* hybridization, we analysed the expression of the Nogo-A protein at different developmental stages.

Nogo-A is expressed in the orofacial complex already at E12.5 during mouse development (figure 9A). At these early stages, Nogo-A is mainly expressed in immature neurons; in particular, its expression is extremely clear in the primordium of the trigeminal ganglion and in the nerve fibres that extend from it (figure 9B, C). Nogo-A positive nerve fibres approach the oral epithelium (figure 9C) but do not approach the developing tooth germs yet; no expression of Nogo-A could be observed in the tooth germ at this stage (figure 9D).

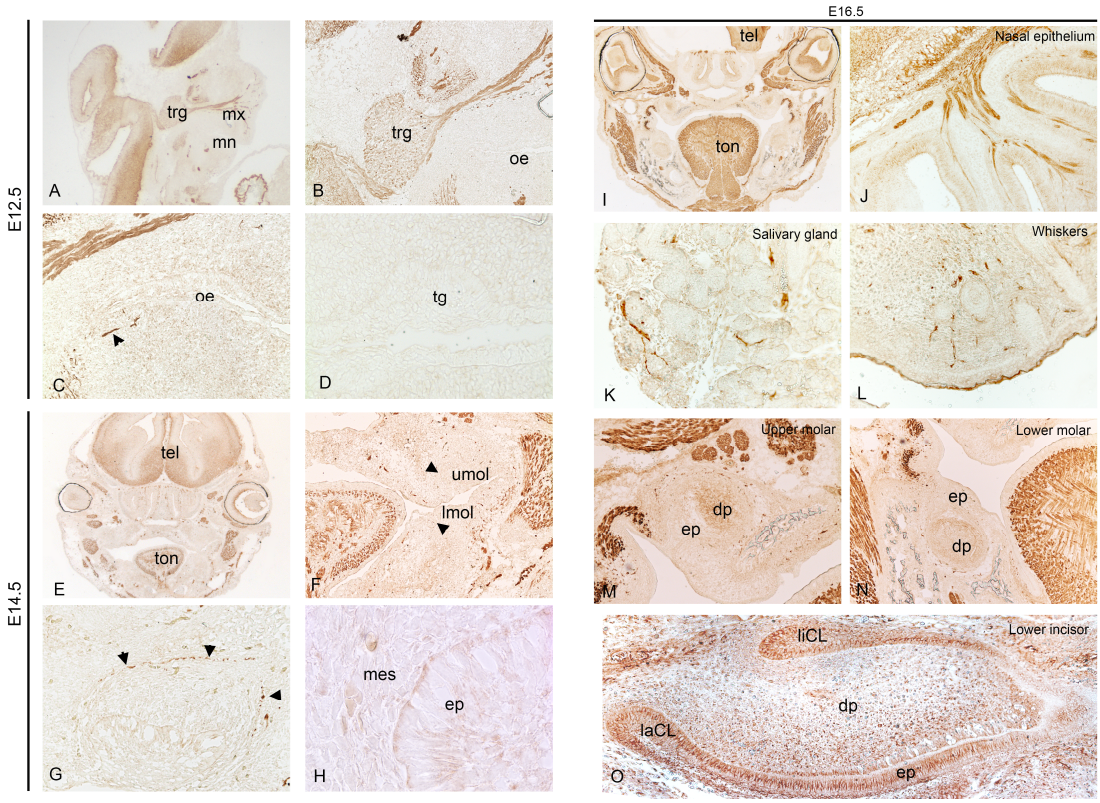
At E14.5, Nogo-A is widely expressed in the orofacial complex (figure 9E). Nogo-A is strongly expressed in the tongue, in muscles, and in nerve fibres approaching and surrounding the developing tooth germs (figure 9E-G. Arrowheads indicate the nerve fibres). At this stage, weak Nogo-A expression can be observed in developing tooth germs (figure 9H).

At E16.5 Nogo-A expression is well established in several orofacial organs (figure 9I). Nogo-A expression is clear in the nasal epithelium and in the nerve fibres innervating it (figure 9J). Nogo-A positive fibres innervate salivary glands (figure 9K), hair follicles and whiskers (figure 9L). From this stage Nogo-A is also consistently expressed in developing first molars and incisors, both in the dental epithelium and in the dental mesenchyme (figures 9M, N, O).

After birth, the expression of Nogo-A is sustained in the odontoblastic and ameloblastic lineage in the molars (figure 9T, U). In the incisor the expression of Nogo-A is peculiar, particularly in the dental epithelium. Nogo-A expression is high in the labial cervical loop (laCL), in mature ameloblasts and in the underlying stratum intermedium (figure 9P, Q, S); it is however definitely inferior in transit progenitors and immature ameloblasts (figure 9R). Nogo-A is also expressed in the dental mesenchyme close to the laCL (figure 9P) and in more differentiated odontoblasts (figure 9S). In ameloblasts, Nogo-A expression appears high at the apical and lateral membranes as well as in the cytoplasm towards the apical side (figure 9V). In addition, Nogo-A is strongly expressed at the interface between ameloblasts and the underlying stratum intermedium.

The expression of Nogo-A in the nerve fibres innervating the orofacial complex already at early developmental stages suggests that Nogo-A might play a role in orofacial innervation. In addition, its expression in the developing teeth and in particular in the incisor leads to the suggestive hypothesis that Nogo-A could have innervation-independent roles in tooth development and physiology.

Figure 9 (following page). Nogo-A expression in the orofacial complex during development. **A-D)** Nogo-A protein expression at E12.5. Nogo-A is strongly expressed in the trigeminal ganglion primordium (A, B) and in the nerve fibres that grow towards the developing maxillary and mandibular processes (A, B, C). Nogo-A is not expressed in the developing tooth germs at this stage. **E-H)** Nogo-A expression at E14.5. Nogo-A is expressed in many organs of the orofacial complex, including tongue and muscles (E). Nogo-A is strongly expressed in the nerve fibres, which at this stage circumvent the developing tooth germs (F, G). Low levels of Nogo-A protein start to be detected at this stage in developing tooth germs (H). **I-O)** Nogo-A protein expression at E16.5. Nogo-A is expressed in the nerve fibres innervating the various orofacial organs (J,K,L). At this stage, Nogo-A is clearly expressed in developing molars (M,N) and incisors (O), in both dental epithelium and mesenchyme. **P-V)** Nogo-A protein expression in teeth at p0. In the lower incisor, Nogo-A is strongly expressed in the laCL and in differentiated ameloblasts (P, Q, S), while its expression is low in preameloblasts (R). Odontoblasts also express Nogo-A (S). At this stage Nogo-A is generally expressed in both ameloblastic and odontoblastic lineages in molars (T, U). In ameloblasts, Nogo-A expression is highly localized (V). Abbreviations: trg – trigeminal ganglion; mx – maxilla; mn – mandibular; oe – oral epithelium; tel – telencephalon; ton – tongue; umol – upper molar; lmol – lower molar; dp – dental papilla; mes – mesenchyme; ep – epithelium; laCL – labial cervical loop; liCL – lingual cervical loop; po – preodontoblasts; pa – preameloblasts; od – odontoblasts; am – ameloblasts.


E12.5
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A
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J
Nasal epithelium
K
Salivary gland
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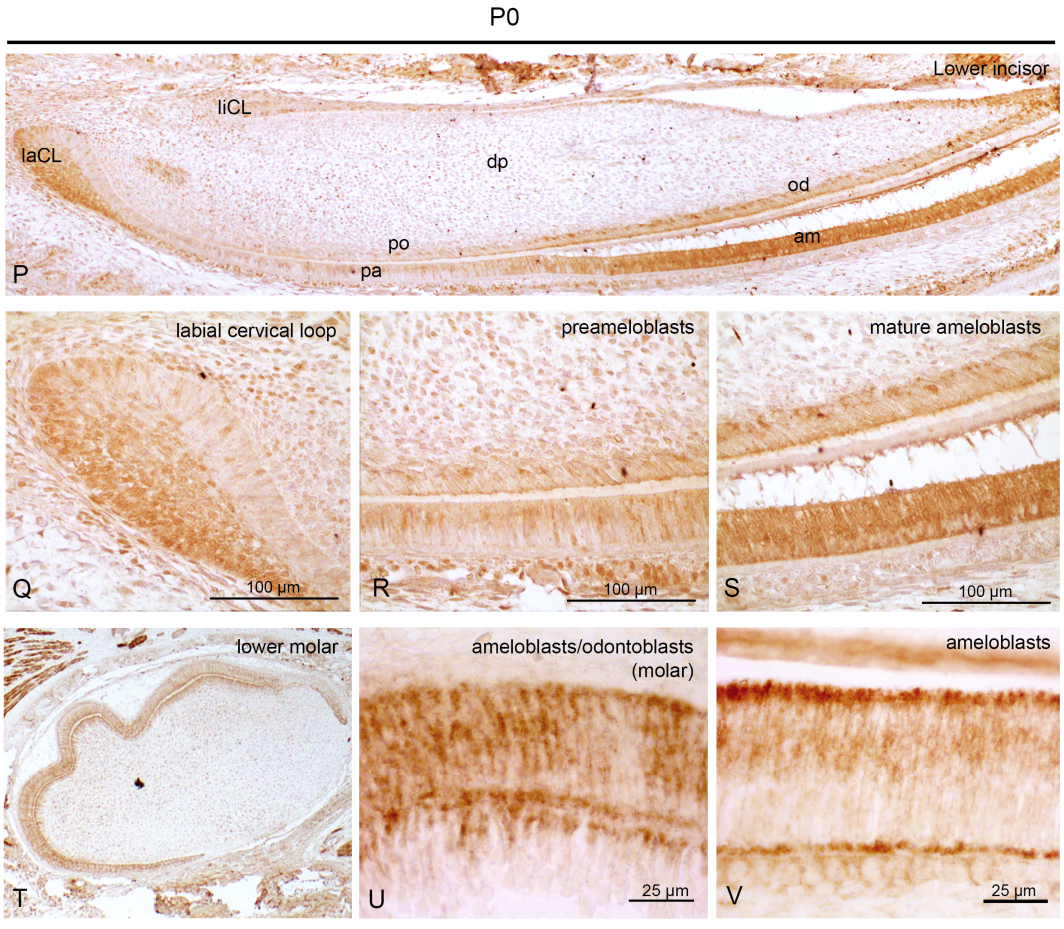
P0
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Lower incisor
liCL
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am
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pa
Q
labial cervical loop
100 µm
R
preameloblasts
100 µm
S
mature ameloblasts
100 µm
T
lower molar
U
ameloblasts/odontoblasts
(molar)
25 µm
V
ameloblasts
25 µm

Nogo-A deletion leads to defective enamel formation in vivo

In order to investigate whether Nogo-A plays an active role in tooth development and innervation we used a transgenic mouse line carrying a genetic deletion at the Nogo locus that specifically impairs the production of Nogo-A (Nogo-A KO), without affecting the other two isoforms Nogo-B and Nogo-C (Simonen et al., 2003).

Nogo-A KO mice do not show critical defects in tooth development; the teeth are present, they are normal at a macroscopical analysis and they are functional in laboratory conditions (figure 10). µCT analysis showed that Nogo-A KO mice have teeth with grossly normal enamel and dentin (figure 10B, C and figure 10E, F).

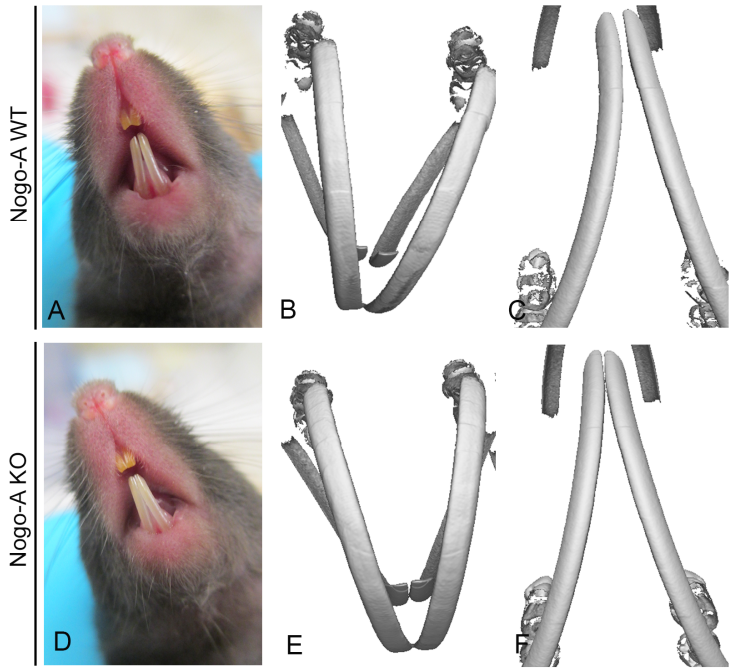


Figure 10. Comparison of wild type and Nogo-A KO teeth. **A,D)** Frontal view of adult mouse teeth, Nogo-A WT (A) and KO (D); **B, E)** µCT comparison of upper incisors of Nogo-A WT (B) and Nogo-A KO (E); C) Nogo-A WT, lower incisors, frontal view, µCT; both upper (B, E) and lower (C, F) incisors have enamel.

At a histological analysis, however, the teeth of new-born Nogo-A KO mice show different tissutal alterations (figure 11). In particular, the ameloblastic layer is strongly disorganized: preameloblasts and ameloblasts form a less compact epithelium, and ameloblast possess a definite tendency to detach from the underlying stratum intermedium (figure 11A-F). Further analyses were performed via transmission electron microscopy (TEM) onto adult specimens, which confirmed and strengthened the main histological observations (figure 11G, H). The structure of the ameloblasts appears completely altered, with their apical regions much less compact and adherent when compared to Nogo-A WT controls. A significant amount of empty areas can be observed in the ameloblast layer; this could be due to the detachment of ameloblasts,

or due to accumulation of exudates in the extracellular compartments. In addition, the adhesion between the ameloblasts and the underlying stratum intermedium is strongly compromised.

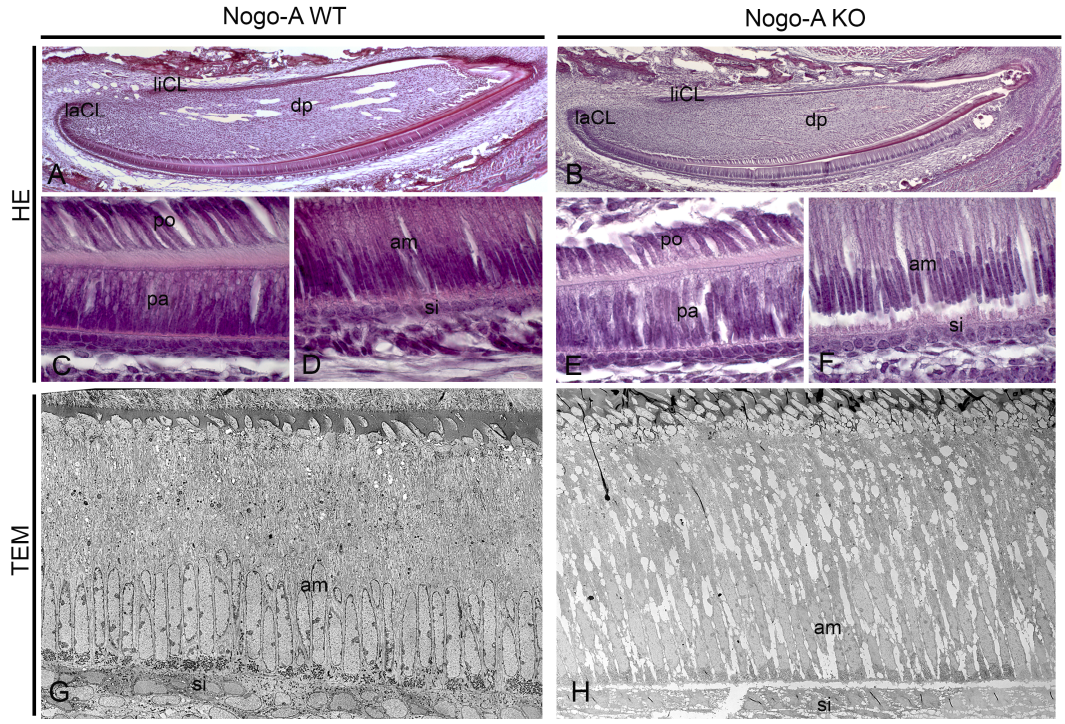


Figure 11. Histological analysis of wild type and Nogo-A KO teeth. **A,B)** The gross morphology of the teeth is not compromised. **C-F)** The dental epithelium of Nogo-A KO mice shows significant morphological alterations. Abbreviations: laCL – labial cervical loop; liCL – lingual cervical loop; dp – dental papilla/pulp; po – preodontoblasts; pa – preameloblasts; am – ameloblasts; si – stratum intermedium.

Following the observation that Nogo-A deletion results in significant defects in ameloblasts, we analysed the structure of the enamel of Nogo-A KO teeth. Classical histology and TEM require decalcification of dental samples in order to obtain tissues slices that can be analysed; these methods, however, do not allow the study of enamel, composed mainly (>90%) of minerals. We therefore used backscattered scanning electron microscopy (SEM) to analyse the fine structure of the enamel of non-decalcified molars and incisors of adult (3 months old) wild type and Nogo-A KO mice. In wild type teeth, the enamel structure is highly organized and compact. The SEM analysis showed an alteration in the fine structure of the enamel of Nogo-A KO teeth. In the lower incisors, the enamel structure is clearly compromised at the point of eruption and towards the tip of the incisor. In wild type incisors enamel a highly spatially ordered disposition of rod and interrod crystals can be observed (figure 12A-C). In Nogo-A KO mice this organization is significantly lost: in particular, rod crystals have highly variable orientation and dimensions (12D-F).

In molars the phenotype is less evident. Nevertheless, rod enamel crystals at the flank of the crown are less strictly organized in Nogo-A KO mice than in wild type controls (figure 12I, L). At the tip of the cusps of Nogo-A KO molars the enamel structure is less compact, with fibrils less tightly packed, which result in apparently more fragile enamel (compare 12H, K).

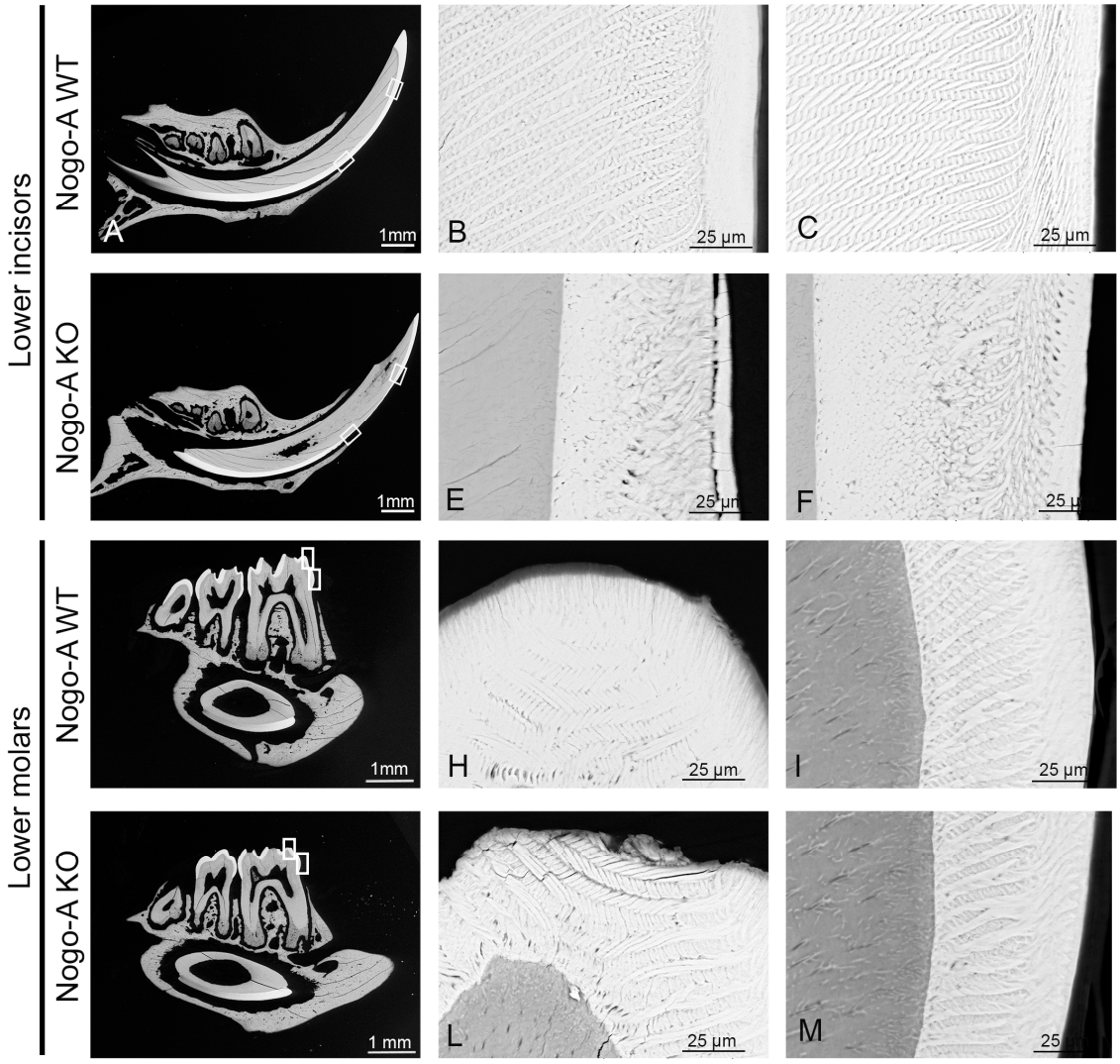


Figure 12. SEM analysis of incisors and molar enamel of wild type and Nogo-A KO adult mice**. A)** Overview of a WT lower incisor and its localization in the lower jaw. **B, C)** Higher magnification of two regions (white boxes in 7A) of WT lower incisor enamel. **D)** Overview of Nogo-A WT lower incisor and its localization in the lower jaw. **E, F)** Higher magnification of two regions (white boxes in 7D) of Nogo-A KO lower incisor enamel. **G)** Overview of WT lower molars and their localization in the lower jaw. **H, I)** Higher magnification of two regions (white boxes in 7G) of WT lower molar enamel. **K)** Overview of Nogo-A KO WT lower molars and their localization in the lower jaw. **L, M**) Higher magnification of two regions (white boxes in 7J) of Nogo-A KO lower molar enamel. Scale bars A, D, G, K: 250µm. Scale bars higher magnifications B, C, E, F, H, I, L, M: 50µm.

We also quantified the relative elemental composition of the enamel of wild type and Nogo-A KO lower incisor using energy-dispersive X-ray spectroscopy (EDS). The composition was analysed at different stages of enamel differentiation, spanning the entire length of the lower incisors. This analysis did not reveal significant alterations in the relative elemental composition of calcium, phosphorus and oxygen, the main components of tooth enamel, in any region. Nogo-A KO mice showed a slightly lower concentration of iron on the surface of the enamel of the most anterior region of the incisor, towards the tip (KO: 2-2,3% of total mass, WT: 3-3,5% of total mass).

Effects of Nogo-A deletion on gene expression in the developing tooth

Based on the expression pattern of Nogo-A and on the effects of its deletion on enamel morphology, we investigated whether Nogo-A deletion affects gene expression in the developing incisor. At birth, Nogo-A is expressed in the laCL (the dental epithelial stem cell niche) and in the differentiated region, mainly in ameloblasts. Nogo-A is also expressed in the dental mesenchyme in similar, although less defined, patterns. We therefore compared the global gene expression of wild type and Nogo-A KO mice separately in the cervical loops area (posterior region) and in the most differentiated area (anterior region) via RNA sequencing and validated the results via real-time PCR.

The RNA sequencing analysis showed that in general a limited number of genes are significantly ($p < 0.05$) deregulated in Nogo-A KO incisors. Nogo-B, which is known to be upregulated in response to Nogo-A deletion in the CNS (Simonen et al., 2003), is not significantly upregulated in Nogo-A KO incisors, neither in the posterior nor in the anterior regions analysed (figure 13, 14). Similarly, gene expression levels of neither NgR1 nor S1PR2 expression, the main receptors of Nogo-A, are deregulated in Nogo-A KO incisors.

The effects of Nogo-A deletion are different in the posterior and in the anterior region of the incisor. In the posterior region the vast majority of deregulated genes are involved in cytoskeletal remodelling and cell adhesion, in accordance with the main signalling pathways linked with Nogo-A in the CNS (figure 13). In this group, precise members of the family of actin and myosin proteins are particularly downregulated. In addition, genes coding for cytoskeletal proteins generally associated with muscular functions are expressed in the tooth and are significantly downregulated in Nogo-A KO posterior regions. Cytoskeletal proteins play fundamental roles in cell migration, adhesion and differentiation, and Nogo-A is known to affect all these processes in the CNS. The downregulation of such genes in the stem cell niche area, together with the Nogo-A expression in the laCL discussed above, suggests that Nogo-A deletion might affect cell adhesion, migration and cell fate determination in the DESCs niche.

Among the few genes deregulated in the Nogo-A KO teeth a significant proportion is constituted by blood vessels-related genes, such as *Vwf*, L*yve1* and different globin genes (figure 13). The downregulation of these genes suggest that Nogo-A KO incisors might display an aberrant vascularization.

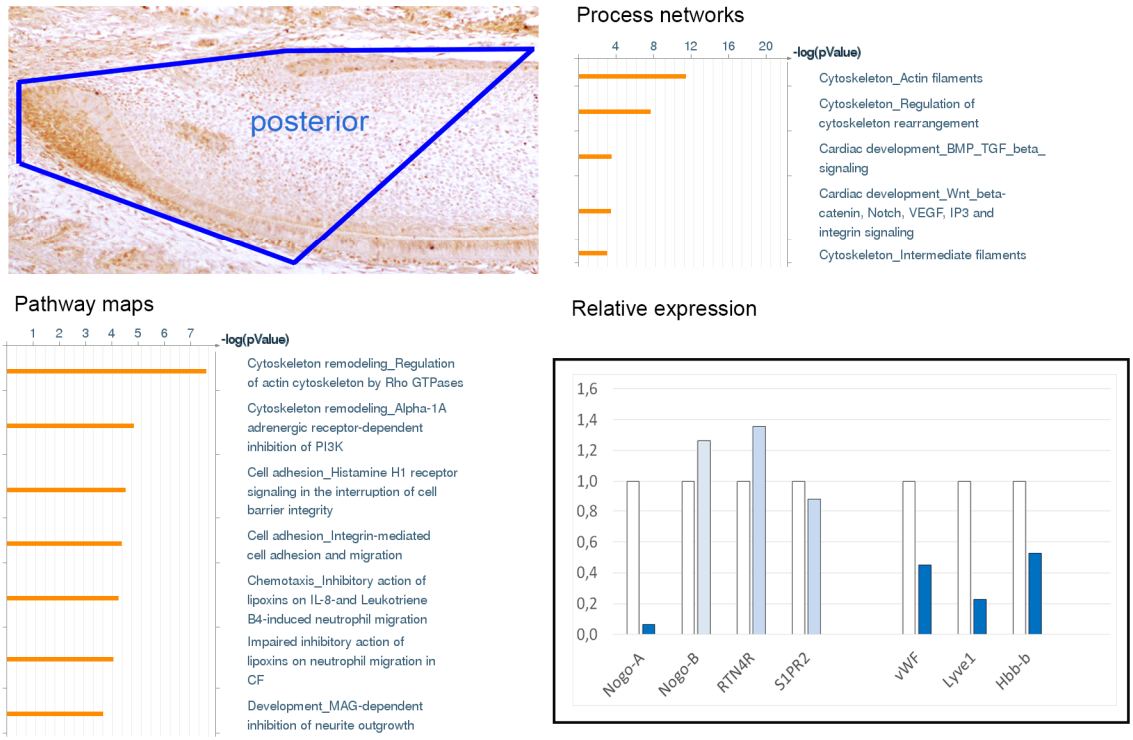
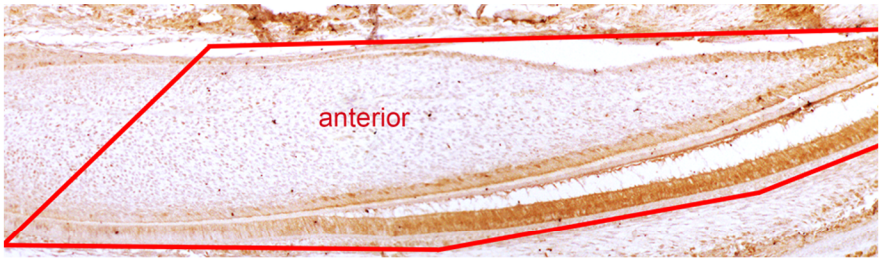


Figure 13. RNA sequencing – posterior region. Top left: region analysed. Top right, low left: process networks and pathways affected by Nogo-A deletion. Low right: Genes coding for Nogo isoforms, receptors and vascularization-associated genes deregulated in the posterior region of the lower incisor following Nogo-A deletion. Light blue columns indicate non-significant fold changes; dark blue columns indicate significant ($p < 0,05$) fold changes.

The expression of only a small proportion of genes is significantly deregulated in the anterior region of Nogo-A KO lower incisors. A subset of these genes codes for proteins involved in protein and vesicular transport, such as dyneins and myosins, and cell adhesion (figure 14) (e.g Galectins 7 and 8 – *lgals 7,8*). Similarly to what observed in the posterior region, a significant proportion of deregulated genes is constituted by blood vessels-related genes, in particular *Hba-a* and *Hbb-b* (figure 14); this observation strengthens the hypothesis that Nogo-A deletion might affect tooth vascularization.

Surprisingly, Nogo-A deletion causes the significant downregulation of the expression of two members of the class of Metallothioneins, *Mt1* and *Mt2* (figure 14). Metallothioneins are involved in the detoxification of heavy metals such as cadmium (Cd), zinc (Zn) and lead (Pb). Exposure to Cd and Pb is known to inhibit proper enamel formation *in vivo* and *in vitro* (Kakei et al., 2009; Gerlach et al., 2000; Tamura et al., 1999).



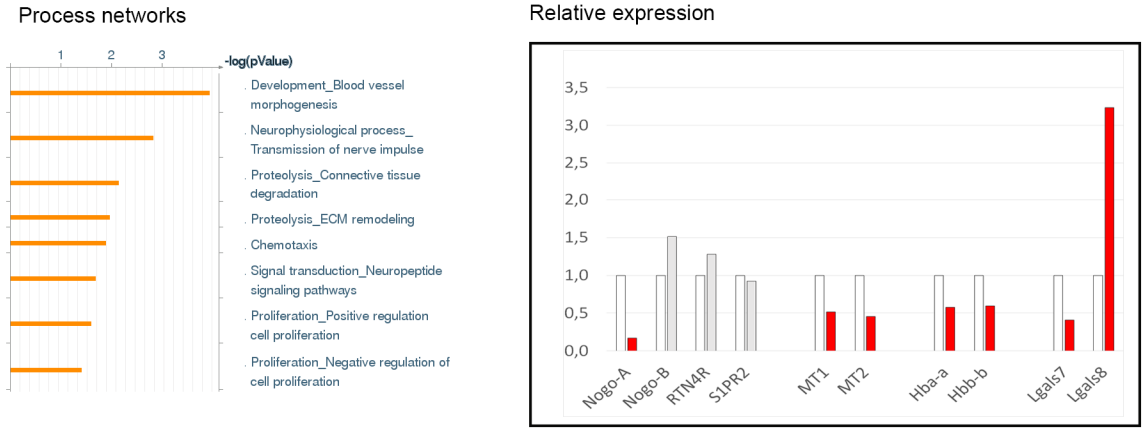


Figure 14. RNA sequencing – anterior region. Top: region analysed. Low left: process networks affects by Nogo-A deletion. Low right: Genes coding for Nogo isoforms and receptors, and genes of interest not included in the categories listed in the network analysis significantly affected following Nogo-A deletion. Grey columns indicate non-significant fold changes; red columns indicate significant ($p < 0.05$) fold changes.

Effects of the deletion of Nogo-A on tooth differentiation markers

We investigated whether Nogo-A deletion affects the localization of proteins involved in tooth cytodifferentiation via immunohistochemistry on wild type and Nogo-A KO specimens from different developmental stages.

Our analysis did not reveal significant alterations in the expression and the localization of the majority of the proteins studied, including Notch1, Notch2, Notch3, β-catenin, DSPP (dental sialophosphoprotein, marker of odontoblastic differentiation; data not shown), and junctional proteins. This result is in accordance with the observed phenotype: significant alterations in the localization and/or the expression of these classes of molecules generally result in much more severe disturbances in enamel formation

Interestingly, in different Nogo-A KO incisors we observed a delayed expression of amelogenin, a key component of the immature enamel matrix (compare figure 15A and figure 15B), along the ameloblastic lineage. In these specimens, amelogenin expression reaches the highest level much more anteriorly than in wild type controls.

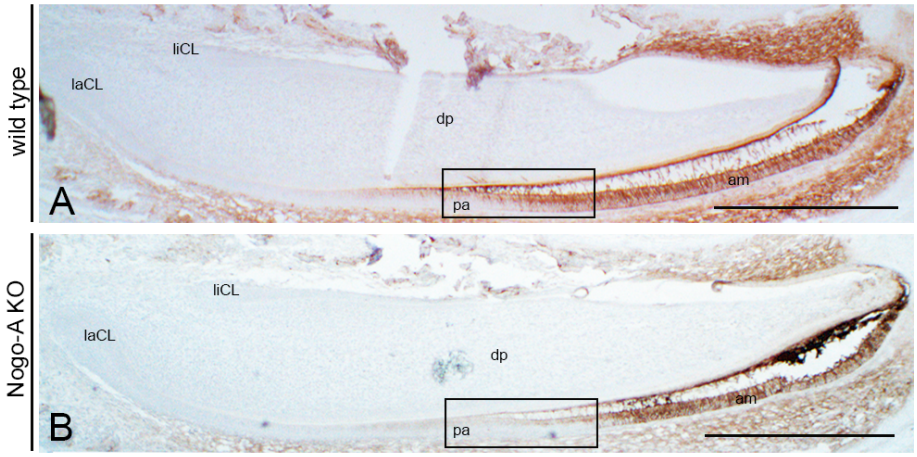


Figure 15. Expression of amelogenin in lower incisors of control (A) and Nogo-A KO (B) newborn mice. Scale bar: 500 µm.

The observed delay in the expression of amelogenin might be an indicator of a more general alteration in the dynamics of stem cells/progenitor migration and differentiation along the ameloblastic lineage.

Blocking of Nogo-A function *in vitro*

The expression of Nogo-A in the mouse incisors and the results of the RNA sequencing suggest that Nogo-A could be involved in the regulation of stem cell adhesion within the niche and their migration along the ameloblastic lineage. In order to investigate whether the blocking of Nogo-A function would affect the functionality of DESCs, we performed tooth organ cultures of mouse lower incisors (isolated from new-born mice). Dissected incisors were cultured on a Trowell grid system and treated with the Nogo-A blocking antibody 11C7. Prior to treatment, we performed a BrdU pulse in order to label stem/progenitor cells and their progeny.

In these conditions, the cervical loop produces new dental epithelium, which can be seen as an upward movement of the apical end of the epithelium to encompass the apical mesenchyme (figure 16). Treatment with the 11C7 Nogo-A blocking antibody leads to an increased growth of the dental epithelium compared to control samples (figure 16, top and figure 16, below). The laCLs of 11C7-treated incisors grow at a definitely higher rate than controls, bending significantly (figure 16, black arrowheads).

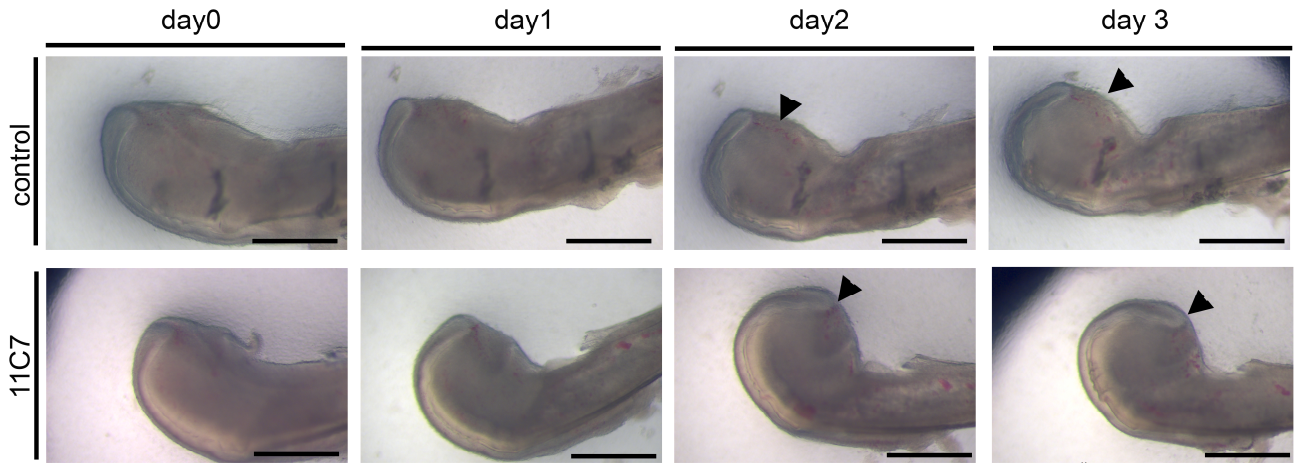


Figure 16. Antibody-mediated blockage of Nogo-A activity in cultured lower incisors (p0). Top row: control. Lower row: incisors treated with the Nogo-A blocking antibody 11C7 (30µg/ml). Scale bar: 400 µm.

We quantified the growth of the dental epithelium by measuring the length of the epithelium included between the laCL and the onset of tooth mineralization (figure 17A). Incisors treated with the 11C7 antibody show an immediate boost in epithelial growth (figure 17B); the growth of the epithelium reaches a plateau between day 3 and day 4 of culture, both in control and 11C7-treated samples. After 4 days of culture, the length of the posterior region of 11C7-treated incisors increase of more than 300 µm (+33% of the initial length), against an approximate growth of 110 µm (+10%) in the untreated incisors (figure 17C).

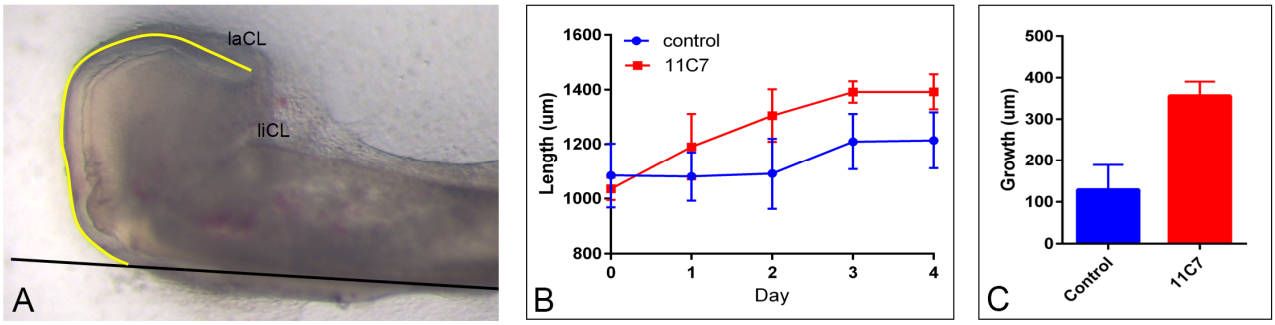


Figure 17. Quantification of the growth of the dental epithelium in control and 11C7-treated incisors. **A)** Definition of measured area (yellow line). **B)** Daily measures of the length of the posterior dental epithelium during the culture period. **C)** Total growth of the measured dental epithelium during the culture period.

In order to understand whether the observed increased growth of the dental epithelium is due to an increased proliferation and differentiation of DESCs, we marked proliferating DESCs within the laCL via BrdU incubation prior to antibody treatment. Immunolabelling of $BrdU^+$ cells after four days of culture showed the localization of label retaining cells within the cervical loop and of their progeny along the dental epithelium. We observed that the increased growth of the dental epithelium induced by the blocking of Nogo-A function is accompanied by a strong increase in the number of $BrdU^+$ cells along the dental epithelium and within the dental mesenchyme (figure 18). In particular, we could observe an obvious increase in the number of newly generated cells migrating out of the stem cell niches. This observation indicates that blockage of Nogo-A function in incisors leads to an increased activation of dental epithelial and mesenchymal stem cells, which results in the observed accelerated growth.

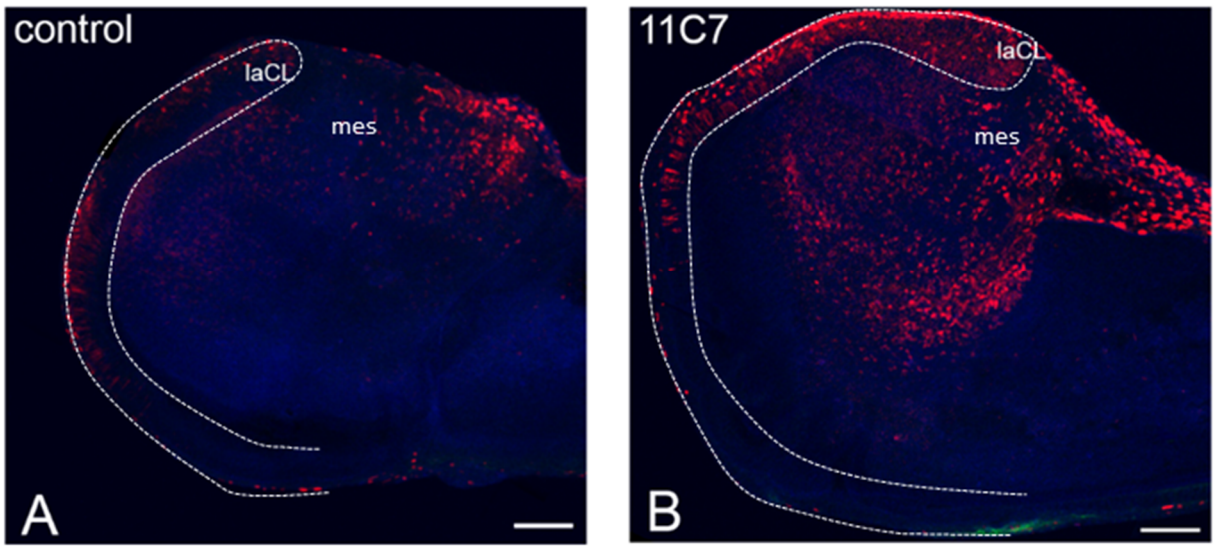


Figure 18. BrdU pulse labelling of stem cells progeny in incisor organ cultures. **A)** Control; **B)** 11C7-treated. The white dotted line shows the dental epithelium. laCL: labial cervical loop; mes: mesenchyme. Scale bar: 100 µm

Effects of Nogo-A deletion on tooth innervation in a microfluidic co-culture system

In order to study the role of Nogo-A in tooth innervation, we performed co-cultures of trigeminal ganglia and tooth germs isolated from wild type and Nogo-A KO mice. In particular, we would like to investigate whether the deletion of Nogo-A would alter the repulsion/attraction balance that developing tooth germs have towards growing nerve fibres.

The main issue of classical co-cultures consists in the fact that different tissues and organs require specific culture media in order to survive and maintain physiological behaviours in culture. We therefore optimized a microfluidic co-culture system: this system allows the culture of trigeminal ganglia and tooth germs each in their own optimal medium and, at the same time, the passage of axons from one culture compartment to the other (Park et al., 2006; Neto et al., 2014; Pagella et al., 2015a, 2014b) (Figure 19A,B,C).

We demonstrated that tooth germs can be effectively cultured for 10 days in this system; in co-culture, tooth germs isolated from different developmental stages maintained *in vitro* the same repulsive-attractant effects on innervation that they show *in vivo* (figure 16D, E)(Pagella et al., 2014b). Embryonic molars (E16.5) efficiently repealed axonal growth, while postnatal molars (P5) attracted innervation from the co-cultured embryonic trigeminal ganglia (figure 19D, E).

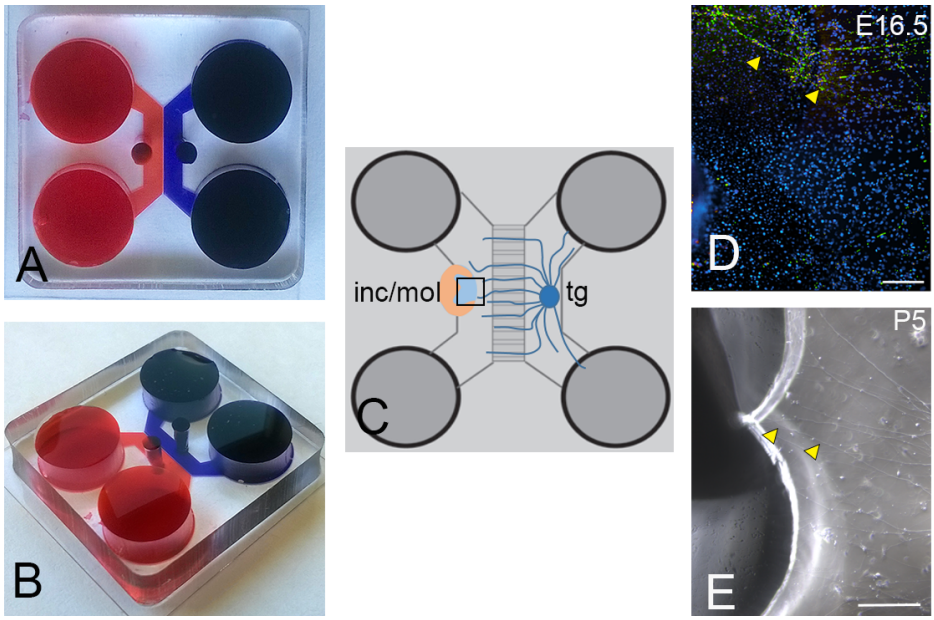


Figure 19. Microfluidic co-culture system for the study of tooth innervation. **A, B)** Overview of the microfluidic co-culture device. **C)** Schematic representation of the co-culture set-up. **D)** Embryonic tooth germs are never contacted by growing trigeminal fibres (yellow arrowheads) in culture. **E)** Postnatal (P5) teeth are quickly innervated by growing trigeminal nerve fibres (yellow arrowheads). Scale bars: D, E) 200 µm

Based on these observations we co-cultured trigeminal ganglia and tooth germs from E16.5 wild type and Nogo-A KO embryos, in order to investigate whether Nogo-A deletion could affect tooth innervation. In

a single plate, we co-cultured single trigeminal ganglia with one wild type and one Nogo-A tooth germ (either molars or incisors; figure 20A). At this developmental stage, wild type teeth actively repeal innervation *in vivo*; consistently with this behaviour *in vivo*, we did not observe any nerve fibre approaching E16.5 wild type tooth germs in up to 10 days of co-culture (figure 20B, C). Also Nogo-A KO tooth germs mainly repeal innervation; however, we could observe in a subset of co-cultures (n = 2/10) clear innervation of Nogo-A KO tooth germs (figure 20D, E). These observations suggest that Nogo-A can influence overall tooth innervation.

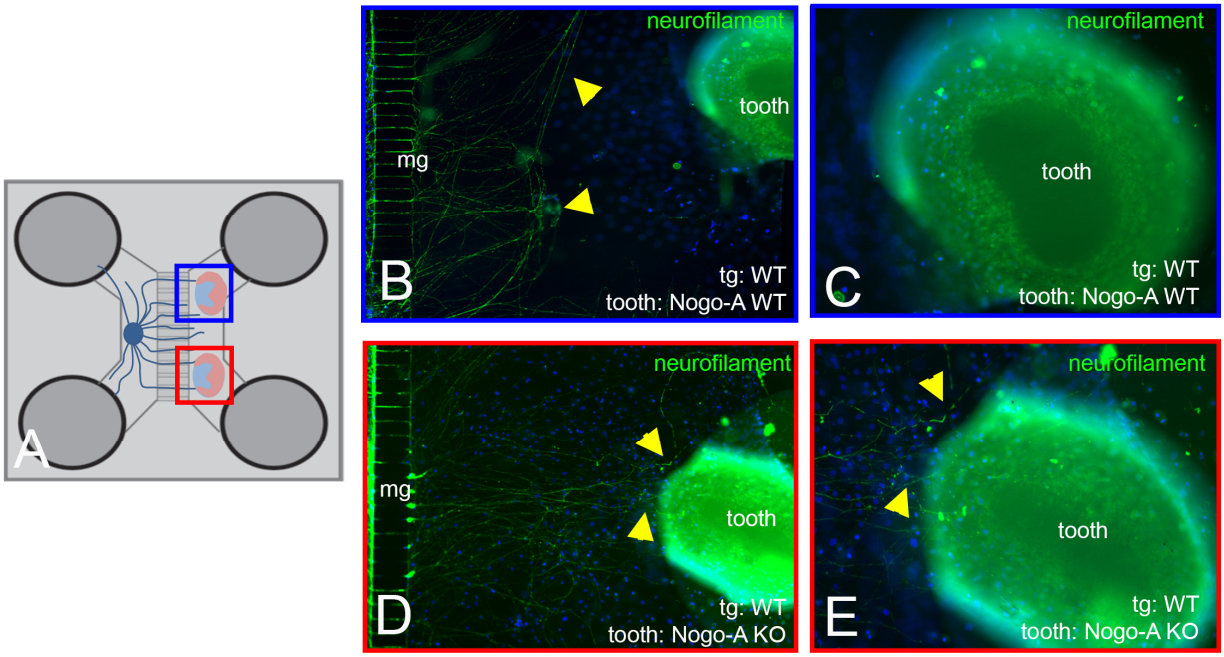


Figure 20. Co-cultures of wild type (WT) and Nogo-A KO trigeminal ganglia and tooth germs. **A)** Co-culture scheme. Blue box: trigeminal ganglion WT, tooth germ WT; red box: trigeminal ganglion WT, tooth germ KO. **B,C)** Immunofluorescence (green: neurofilament) showing innervation of wild type E16.5 tooth germs in vitro: neurites (yellow arrowheads) do not approach the tooth germ. **D,E)** Immunofluorescence (green: neurofilament) showing innervation of Nogo-A KO E16.5 tooth germs in vitro: neurites (yellow arrowheads) can be observed in close proximity to the tooth germ.

Nogo-A deletion does not impair incisors re-growth upon injury

Mouse incisors grow continuously throughout the life of the animal. Based on the observations that Nogo-A is expressed in the laCL of the mouse incisor, that its deletion leads to the formation of defective enamel, and that blocking of Nogo-A *in vitro* increases the generation of new dental epithelium, we investigated whether the genetic deletion of Nogo-A would alter the ability of mouse incisors to regrow following severe injury. We therefore trimmed the anterior region of one of the lower incisors of wild type and Nogo-A KO mice, and followed the regrowth of the incisor. Both wild type and Nogo-A KO mice could regrow the damaged incisors within 4 days from the day of the injury (figure 21). We did not observe a significant difference in the regrowth rate of the mouse incisor.

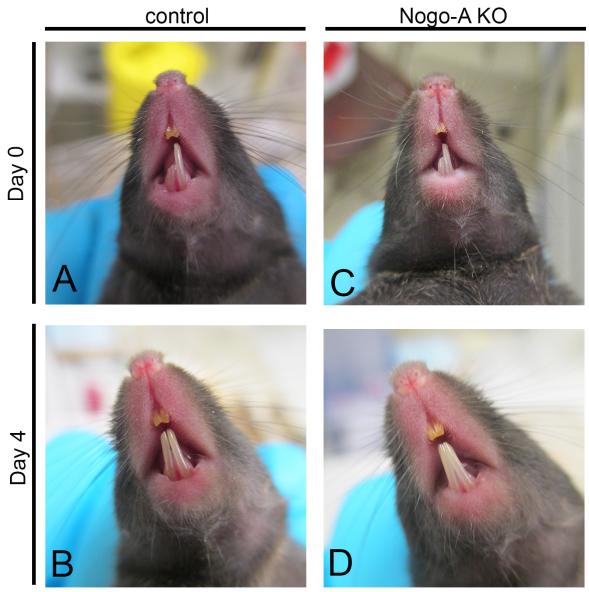


Figure 21. Tooth regeneration following injury in wild type and Nogo-A KO mice. A, B) Wild type; C, D) Nogo-A KO.

7. Discussion

In the last decades Nogo-A has emerged as one of the most promising molecule for the regeneration of the CNS (Schwab, 2010; Wahl et al., 2014; Freund et al., 2006; Schmandke et al., 2014). The various roles of Nogo-A during CNS development, homeostasis, and regeneration have been intensely investigated. Surprisingly, although the expression of Nogo-A outside the CNS was observed in different studies in the past (Osborne et al., 2004; Al Halabiah et al., 2005; Lee et al., 2012), almost no data exists regarding the non-neuronal functions of Nogo-A. Therefore, we have performed the first profound analysis concerning the roles of Nogo-A in tissues other that the CNS.

Nogo-A is widely expressed in the orofacial regions from the earliest developmental stages in the mouse. Consistently with the observations in the CNS, Nogo-A is expressed in the developing nerve fibres that will constitute the cranial nerves. In addition, Nogo-A is expressed in a plethora of orofacial tissues, such as muscles, salivary glands, hair follicles and teeth. In teeth, Nogo-A is expressed by both dental epithelium and mesenchyme well before the onset of tooth innervation, thus suggesting that Nogo-A might be also involved in tooth development. Only one study has shown previously that *Nogo* is expressed in developing human teeth (Al Halabiah et al., 2005).

Nogo-A expression in teeth is correlated with the differentiation status of the inner enamel epithelium. In the incisor, Nogo-A is expressed in the labial cervical loop (the dental epithelial stem cell niche) and in differentiated ameloblasts (highly polarized cells responsible for the production of enamel), while its expression is very low in transit progenitors and pre-ameloblasts. This expression pattern suggests that Nogo-A might be involved in ameloblast differentiation and function, as well as in epithelial stem cells behaviour.

Nogo-A deletion leads to defective enamel formation

In order to investigate the role of Nogo-A in tooth development we analysed mice carrying a selective deletion of the Nogo-A transcript (Nogo-A KO) (Simonen et al., 2003). This deletion selectively ablates Nogo-A, while leaving the other splicing isoforms of the *Nogo* gene unaffected. Thorough analysis of these mice showed that the absence of Nogo-A leads to defective enamel formation. Electron microscopy analysis showed an altered organization of enamel fibrils that seriously compromises enamel structure. The alteration in enamel is may occur due to the clear disorganization of the ameloblastic layer that was observed in the Nogo-A KO mice. Indeed, the overall appearance and structure of ameloblasts in the mutant mice is seriously compromised and, furthermore, ameloblasts detach from each other and from cells of the overlying stratum intermedium layer.

Enamel formation requires regulated secretion of enamel proteins, involves a specific mineralization process, and activates a mechanism of enamel proteins reabsorption by ameloblasts. Alterations in each of these processes can lead to the generation of defective enamel. Amelogenesis Imperfecta (AI) is the term that designates hereditary developmental malformations of the enamel in humans. AI includes a plethora of conditions that derive from alterations during all stages of enamel formation (Mitsiadis and Luder, 2011; Wright, 2006; Poulsen et al., 2008; Crawford et al., 2007). Disturbances in ameloblasts at their secretory stage lead to the formation of a thinner enamel, which is indicated by the term of hypoplastic AI. This quantitative deficiency can vary significantly from the less severe smooth to the severe rough forms of hypoplastic AI. Disturbances in the mineralization and/or the maturation step of enamel development result in hypomineralized AI, which is characterized by a qualitative deficiency of enamel. The enamel in the hypomineralized AI exhibits the normal thickness but its content in minerals is reduced while being increased in proteins. A moderate reduction in enamel mineralization leads to the hypomaturated type of AI, while a more severe mineral deficiency and protein retention results in the hypocalcified form of AI (Wright, 2006; Hu et al., 2007; Mitsiadis and Luder, 2011). So far, the different forms of AI have been associated to mutations of genes coding for enamel matrix proteins such as amelogenin (AMELX) and enamelin (ENAM) (Rajpar et al., 2001; Gibson et al., 2001), as well as enzymes and proteins such as enamelysin (MMP20) (Wright et al., 2009) and kallikrein-4 (KLK4) (Hart et al., 2004), which are required for enamel matrix degradation during the maturation phase (Bartlett, 2013).

However, neither evident reduction in the enamel thickness nor significant changes in enamel composition were obvious in teeth of the Nogo-A KO mice. Surprisingly, the enamel of Nogo-A KO mice is characterized by an alteration in the structure and the disposition of the enamel rods that is not associated with a reduction of mineralization or a decrease in enamel thickness. To our knowledge, such a defect in the enamel structure has not been reported yet. Hence, it appears that Nogo-A regulates enamel development through a molecular mechanism that differs from the ones already characterized by studying the classical forms of AI.

The disorganization that has been observed in the ameloblastic layer of Nogo-A KO mice may indicate severe functional defects of ameloblasts that could interfere with their secretory and reabsorption capacities. The dysfunction of ameloblasts could be due the disorganization of their cytoskeleton, which is the main target of Nogo-A signalling in neuronal cells (Schmandke et al., 2014). Indeed, both protein exocytosis and endocytosis events depend on cytoskeleton integrity (Anitei and Hoflack, 2011). Therefore, cytoskeletal dysfunction following the Nogo-A deletion could lead to significant changes in protein export and reabsorption in ameloblasts, which would finally result in the production of defective enamel. RNA sequencing analysis that we performed in Nogo-A KO incisors has shown a deregulation in the expression of genes that code for proteins involved in connective tissue degradation and extracellular matrix remodelling. These results reinforce our hypothesis that Nogo-A deletion interferes with enamel matrix

degradation during the maturation stage. However, genes that are known to affect enamel maturation processes such as *Mmp20* and *Klk4* were not deregulated according to this analysis.

Interestingly enough, RNA sequencing analysis has shown that Nogo-A deletion caused a significant downregulation of two genes coding for the metallothionein Mt1 and Mt2 proteins. Although the physiological function of metallothioneins is still unclear, previous studies have suggested that these molecules may be involved in heavy metal detoxification and formation of enamel defects. Indeed, it has been shown that metallothioneins sequester environmental contaminants such as cadmium (Cd), mercury (Hg) and lead (Pb), and that exposure to these elements induces the expression of genes coding for metallothioneins (Tamura et al., 1999). Furthermore, it has been shown that Cd and Pb strongly impair enamel development both *in vitro* (Gerlach et al., 2000) and *in vivo* (Bawden and Hammarstrom, 1975; Tamura et al., 1999; Kakei et al., 2009), mainly by interfering with dental enamel matrix metalloproteases. Taken together these findings suggest that the decreased levels of metallothioneins in the Nogo-A KO teeth may result in a reduced ability to detoxify heavy metals such as Cd and Pb. Consequently, increased levels of heavy metals in the enamel of mutant mice might lead to the observed enamel defects.

Nogo-A functions on dental stem/progenitor cells

The mouse dentition is composed of two tooth types: molars and incisors. Differently from human teeth and mouse molars, mouse incisors are continuously growing. Their continuous growth ensures replenishment of enamel, which is lost due to their mastication habits. Enamel production is sustained by the continuous generation of ameloblasts from the labial cervical loop (laCL), which is a niche hosting dental epithelial stem cells (DESCs)(Harada et al., 1999). DESCs migrate from the laCL anteriorly towards the tip of the incisor and differentiate into all dental epithelial lineages (figure 23). Through this continuous process, ameloblasts and enamel are regenerated unceasingly throughout the life of the animal. In parallel, dental mesenchymal stem cells (DMSCs) located in proximity to the laCL contribute to the regeneration of the dental mesenchyme (figure 23; (Zhao et al., 2014a)). DESCs and DMSCs have been identified using a combination of label-retaining assays and genetic lineage tracing using different markers (Harada et al., 1999; Seidel et al., 2010; Juuri et al., 2012; Zhao et al., 2014a). DESCs were initially localized in the labial cervical loop by tracing via retaining of radioactive thymidine, bromodeoxyuridine and fluorescent lipophilic dyes (Smith, 1980; Harada et al., 1999). The first genetic markers for DESCs and DMSCs were identified by genetic lineage tracing. In particular, the expression of Sox2 (Juuri et al., 2012) and Bmi1 (Biehs et al., 2013) characterizes the DESCs from the labial cervical loop of the mouse incisor. In addition, it was demonstrated that both dental epithelium and dental mesenchyme are completely reconstituted by two populations of Shh-responsive $Gli1^{+}$ stem cells located in the labial cervical loop and in the mesenchyme immediately

adjacent to it, respectively (Seidel et al., 2010; Zhao et al., 2014a), thus indicating that Gli1 expression marks stemness in both compartments.

Nogo-A expression in the labial cervical loop and in the dental mesenchyme of postnatal incisors (figure 9) indicate that Nogo-A could play a role in the behaviour of both dental epithelial and mesenchymal stem cells. It is known that Nogo-A regulates differentiation of neural stem cells and the migration of neuroblasts within the subventricular zone, an adult neurogenic niche (Rolando et al., 2012). However, nothing is known about the role of Nogo-A in other stem cell niches.

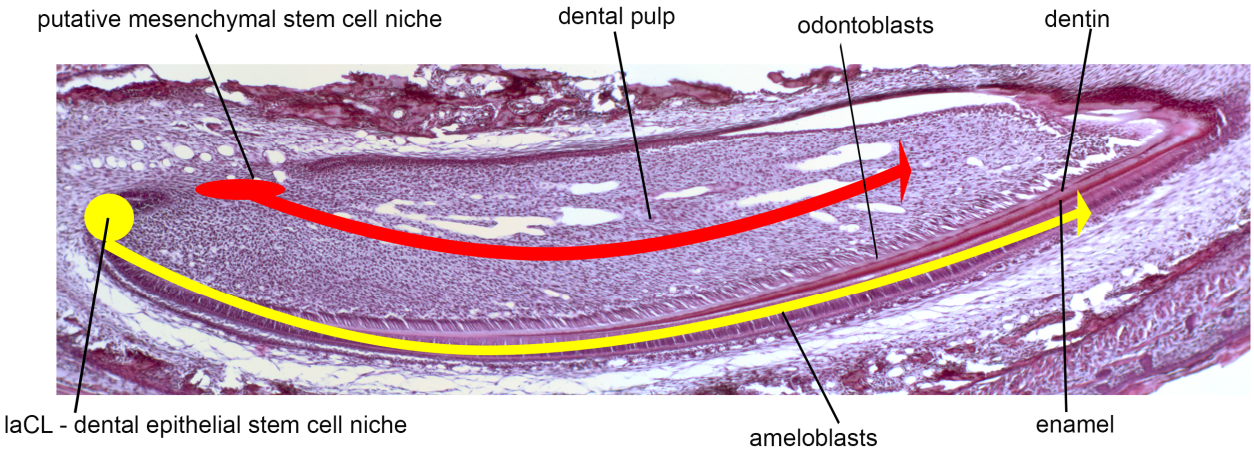


Figure 23. Representation of the main components of the rodent incisor at birth (p0). The dental epithelial stem cell niche, the labial cervical loop (laCL), is marked by a yellow circle; the yellow arrow highlights the anterior migration of the progeny of dental epithelial stem cells. A red ellipse marks the putative mesenchymal stem cell niche, and the migration of the cell originated from this region is indicated by the red arrow.

RNA sequencing analysis performed on Nogo-A KO incisors showed a strong deregulation in the expression of genes coding for proteins of the cytoskeleton, cell adhesion, and cell migration. These groups of proteins are essential for processes regulating stem cell niche homeostasis and stem/progenitor cells migration out of the niches (Chen et al., 2013). Therefore, deregulation of the expression of these genes in stem cell niches may indicate an unexpected role for Nogo-A in regulating stem cell function and their migration before differentiating into ameloblasts. Since the developmental context of enamel formation is time and space dependent, enamel defects due to Nogo-A deletion might be caused by an early effect affecting the behaviour of DESCs within the niche. A similar effect might be also observed in the mesenchymal compartment of incisor that would affect the physiology of mesenchymal stem cell niche.

To test this hypothesis, incisors isolated from new-born wild type mice were treated with a Nogo-A blocking antibody (11C7) and cultured *in vitro*. In these culture conditions, mouse incisors can be maintained for several days and their development mimics well the *in vivo* situation (Harada et al., 1999). The epithelial growth was assessed daily by measuring the regression of the cervical loop towards the posterior end of the incisor. The blocking of the Nogo-A activity resulted in the striking increase of the

dental epithelium. The difference in the growth rate between treated and non-treated cervical loops was particularly evident in the first two days of culture. In a second set of experiments, dental epithelial and mesenchymal stem/progenitor cells were labelled via a BrdU pulse treatment. A three-dimensional localization of dental epithelial and mesenchymal stem cells and their progenies was monitored via whole mount immunolabelling of $BrdU^+$ cells after 4 days of culture. The results showed a striking increase in the amount of cells generated by both epithelial and mesenchymal stem/progenitor cells in 11C7-treated incisors when compared to the wild-type incisors. Indeed, a higher number of $BrdU^+$ cells were found not only within the stem cell niches, but also in the most anteriorly located dental mesenchyme and epithelium (differentiated territories of the tooth). These results clearly demonstrate that Nogo-A is involved in the regulation of both epithelial and mesenchymal stem cells behaviour in the developing mouse incisors. Increased stem cell proliferation after blocking the Nogo-A activity results in an amplified migration of not yet differentiated cells outside of the stem cell niches territory, which contributes to the increased growth of the incisors.

Conserved signalling pathways such as Fgf, Bmp, Tgf-β, Shh, and Notch regulate the homeostasis and the proliferation of the epithelial and mesenchymal stem cells in the cervical loop area (Tummers and Thesleff, 2009; Juuri et al., 2013; Zhao et al., 2014b; Seidel et al., 2010). The regulation of these signalling pathways affects cervical loop size and hard tissue formation, as well as the symmetry of incisors (Juuri et al., 2012; Plikus et al., 2005; Wang et al., 2009; Pouyet and Mitsiadis, 2000). Surprisingly, RNA sequencing analysis in Nogo-A KO incisors did not reveal significant alterations in the expression of any member of these pathways. It is thus tempting to speculate that Nogo-A could influence dental stem cell behaviour independently of these factors by signalling directly on the cytoskeleton and downstream molecules affecting cell adhesion, fate choice, and migration. It is worth noting that Nogo-A expression in both the laCL and transit amplifying progenitors (TAP) zone of incisors is similar to that observed for E-cadherin, which regulates DESCs exit from their niche (Li et al., 2012). Moreover, a similar effect observed following Nogo-A neutralization has been reported after deletion of E-cadherin that led to the massive exit of DESC from the laCL niche (Li et al., 2012). Therefore, Nogo-A might directly regulate stem cell egress from their niche either by controlling cytoskeletal modifications or by interacting with cell adhesion molecules such as cadherins and integrins (Schwab, 2010).

Taken together, these results indicate that Nogo-A deletion/neutralization stimulates the proliferation of both dental epithelial and mesenchymal stem cells and their egress from the niches. This effect is not associated with alterations in the expression of genes coding for proteins of the major signalling pathways, which regulate the fate, proliferation and differentiation of dental stem cells. This leads to the hypothesis that Nogo-A regulates stem cell behaviour downstream of these factors, thus affecting directly the polarity, adhesion and migration of stem cells. Nogo-A could contribute to the interactions between stem and

stromal cells within the niches, regulating their fate and migration (figure 24). Inhibition of Nogo-A signalling would affect these interactions, thus disturbing the normal growth of the incisor.

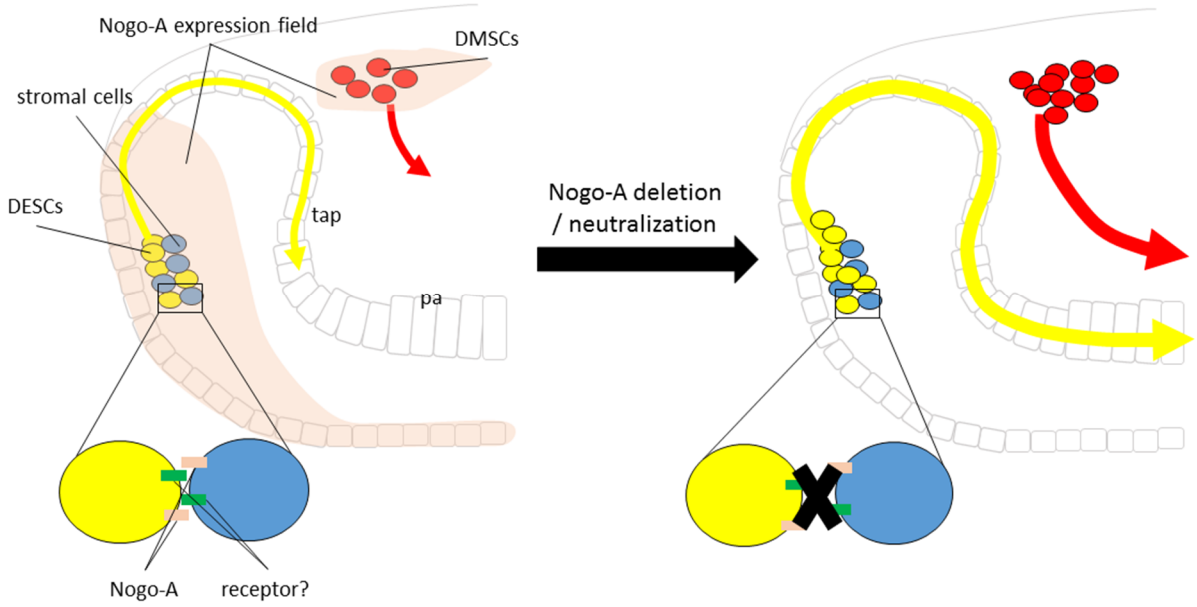


Figure 24. Hypothetical model of Nogo-A function in the mouse incisor. Nogo-A is expressed in the labial cervical loop and by dental mesenchymal stem cells mediating the stem cell-niche interaction. When Nogo-A is neutralized or deleted, stem cells increase their proliferation and, consequently, they give rise to an increased number of daughter cells (larger and longer arrows in the right panel). DESC: dental epithelial stem cells; DMSCs: dental mesenchymal stem cells; tap: transit amplifying progenitors; pa: preameloblasts.

Nogo-A deletion affects innervation of developing tooth germs

Innervation is essential for the development, homeostasis and regeneration of all tissues and organs (Pagella et al., 2014a). Unravelling the molecular mechanisms that underlie innervation and re-innervation of the various organs is therefore fundamental in particular in the light of regenerative medicine (Pagella et al., 2014a). Since Nogo-A is a crucial regulator of the development, maturation and regeneration of the nervous system (Schwab, 2010) we investigated whether Nogo-A deletion could affect tooth innervation in a microfluidic co-culture system. This system allows the co-culture of neurons/ganglia with tissues/organs in their perspective culture media, while allowing the contact between axon terminals and their target tissues (Pagella et al., 2014b). In these *in vitro* culture devices, tooth germs from different developmental stages maintained the same repulsive-attractant effects on trigeminal innervation that was observed *in vivo* (Pagella et al., 2014b). For example, tooth germs isolated from embryonic stages actively repeal trigeminal innervation *in vitro* (Pagella et al., 2014b).

In the last decades a plethora of neurotrophic and neurorepulsive signalling molecules have been shown to regulate tooth innervation (Pagella et al., 2014a). These molecules affect the timing and abundance of tooth innervation to a different degree. To check the suggested repulsive function of Nogo-A during tooth

development we performed co-cultures of trigeminal ganglia and developing tooth germs isolated from E15.5 Nogo-A KO mouse embryos. In a subset of experiments (20%), axons emanating from the trigeminal ganglia approached and contacted the tooth germs. In contrast, such contacts were never established when trigeminal ganglia were co-cultured with tooth germs of wild type mice. These results confirm that Nogo-A may act as a repulsive signal during embryonic stages of odontogenesis.

No obvious difference could be identified in the innervation of wild type and Nogo-A KO teeth *in vivo* by immunohistochemistry (data not shown). RNA sequencing analysis performed on Nogo-A KO incisors did not reveal any difference in the expression of genes coding for neurotrophic or neurorepulsive factors, thus suggesting that Nogo-A deletion does not affect the general innervation of teeth *in vivo.* It is however possible that Nogo-A could affect the fine regulation of nerve fibres growth and branching within teeth.

The above-mentioned findings represent the first thorough analysis of the role of Nogo-A outside the CNS, and indicate important and unexpected functions of this molecule that could be taken into account for the needs of regenerative medicine.

"Out of the brain": completing the knowledge of Nogo-A functions outside the CNS

The observation that Nogo-A regulates stem cell function in teeth raises the possibility that it could play similar roles in stem cell niches of other organs and tissues. The main target of Nogo-A signalling, the RhoA-ROCK pathway, is fundamental in cytoskeletal modifications, adhesion, migration, proliferation and differentiation (including polarization) of virtually every cell type (Schmandke et al., 2013, 2007; Otsu et al., 2011; McBeath et al., 2004; Guilak et al., 2009; Ridley and Hall, 1992; Spiering and Hodgson, 2011). Nogo-A might thus represent a widespread regulator of stem cell behaviour in a variety of organs. It would be therefore important to investigate whether Nogo-A is expressed in other stem cell niches and whether the inhibition of its function may lead to alterations in the fate and kinetics of stem cells. It is worth noting that Nogo-A is expressed in the developing whiskers and hair follicles of the face.

Further studies are needed to identify the exact mechanisms through which Nogo-A regulates stem cell function. In particular, it is not known whether Nogo-A is expressed by stem or stromal cells, or both, within the niche. Moreover, the exact dynamics of cell proliferation, migration, and differentiation following Nogo-A neutralization are not clear yet. This aspect is extremely important, since it is known that the effects of Nogo-A on cell migration and differentiation change significantly depending on the molecular and cellular environment (Schmandke et al., 2013; Rolando et al., 2012; Mingorance-Le Meur et al., 2007; Yan et al., 2012; Su et al., 2007). The new role of Nogo-A as a fine regulator of stem/progenitor cells behaviour in organs other than teeth could broaden enormously the potential of Nogo-A as a therapeutic target for the treatment of various diseases. Clinical trials targeting Nogo-A in the CNS have so far confirmed the absence of side effects following its neutralization (Silver et al., 2014). Nogo-A could represent an extremely promising molecule for the fine-tuning of stem cell function *in vivo* and thus greatly contribute to the development of new regenerative therapies.

Concerning the role of Nogo-A in amelogenesis, it would be interesting to investigate whether the *in vivo* administration of the neutralizing Nogo-A antibody in mouse embryos would lead to more severe defects in enamel and broader alterations in gene expression than these observed in the Nogo-A KO mice. Indeed, the *in vitro* effects of Nogo-A neutralization would suggest a more severe enamel phenotype in the Nogo-A KO teeth *in vivo.* However, according to the observations in the CNS, it is possible that the effects mediated by the Nogo-A neutralization antibody would be more intense than its constitutive deletion (Kim et al., 2003; Simonen et al., 2003). Indeed, Nogo-A deletion leads to moderate and controversial phenotypes in the CNS when compared to the *in vivo* antibody-mediated Nogo-A neutralization effects (Kim et al., 2003; Simonen et al., 2003; Zheng et al., 2003; Cafferty et al., 2010; Kempf et al., 2013). These discordances are attributed to differences in Nogo deletion mutants, mouse strain genetic background effects, or possible compensations by other Nogo isoforms (Kempf et al., 2013; Schwab, 2004).

Often the issue of innervation of organs and tissues and their re-innervation following injuries is neglected, leading to unsatisfactory functional recoveries. The discovery of new molecules and/or targets that could facilitate tissue innervation is therefore a key step towards successful regenerative therapies. Therefore, additional *in vivo* and *in vitro* experiments will be needed to elucidate the roles of Nogo-A in the regulation of innervation of the developing and regenerating teeth as well as of dental stem cell niches.

8. Abbreviations

Am: ameloblasts

Ambn: ameloblastin

Amelx: amelogenin

Barx1: barx homeobox 1

Bdnf: brain-derived neurotrophic factor

Bmp: bone morphogenetic protein

BMM: bone marrow macrophages

BrdU: 5-bromo-2'-deoxyuridine

Cdc: cell division cycle

CRMP: collapsin response mediator protein

CNCC: cranial neural crest cells

CNS: central nervous system

DEJ: dentino-enamel junction

Dlx: distaless homeobox

Dmp: dentin matrix protein

Dp: dental pulp

Dspp: dentin sialophosphoprotein

EDS: energy-dispersive X-xay spectroscopy

EDTA: ethylenediaminetetraacetic acid

Enam: enamelin

Ep: epithelium

ER: endoplasmic reticulum

ERM: epithelial rests of Mallassez

Fgf: fibroblast growth factor

GPCR: G protein-coupled receptor

GSK3β: glycogen synthase kinase 3β

Hb: haemoglobin

HERS: Hertwig's epithelial root sheath

IEE: inner enamel epithelium

Islet: ISL LIM homeobox

Klk4: kallikrein-related peptidase 4

laCL: labial cervical loop

Lgals: galectin

Lhx: LIM homeobox

liCL: lingual cervical loop

LINGO: leucine rich repeat and Ig domain containing 1

LTD: long term depression

LTP: long term potentiation

Lyve: lymphatic vessel endothelial hyaluronan receptor

Mes: mesenchyme

Mmp20: matrix metalloproteinase 20

Mn: mandibula

Msx: msh homeobox protein

Mt: metallothionein

Mx: maxilla

Ngf: nerve growth factor

NgR1: nogo receptor 1

NT: neurotrophin

OE: oral epithelium

OEE: outer enamel epithelium

P75NGFR: nerve growth factor receptor

Pa: preameloblasts

PBS: phosphate buffered saline

pCREB: phosphorylated cyclic AMP response element binding

PDL: periodontal ligament

PFA: paraformaldehyde

Po: preodontoblasts

Rac1: Ras-related C3 Botulinum Toxin Substrate 1

RANKL: receptor activator of nuclear factor kappa-B ligand

RhoA: Ras Homolog Family Member A

ROCK: Rho-associated coiled coil containing protein kinase 1

Rtn4: reticulon 4

Rtn4R: reticulon 4 receptor

S1PR2: sphingosine-1-phosphate receptor 2

SEM: backscattered scanning electron microscopy

Shh: Sonic Hedgehog

SI: stratum intermedium

SR: stellate reticulum

TEM: transmission electron microscopy

TG: trigeminal ganglion

TNF: tumor necrosis factor

Ton: tongue

TROY: tumor necrosis factor-α receptor superfamily member 19

vWF: von Willebrand Factor

Wnt: vertebrate homologue of the Drosophila Wingless gene

9. Curriculum Vitae

Personal information:

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Work experience

07/2011 – 11/2011: AIRC research assistant at the University of Milano-Bicocca, group of Prof. Silvia K. Nicolis. "Preparation and study of neural tumor stem cell cultures obtained from mice carrying a conditional mutation of the Sox2 gene".

Education and Training:

12/2011 – currently: PhD Student – ETH/University of Zurich Molecular Life Sciences PhD Program, University of Zurich (Switzerland). "The role of Nogo-A in orofacial development and regeneration" Supervisor: Prof. Dr. Thimios Mitsiadis

10/2008 – 07/2011: Laurea Magistrale – Master Degree in Biology (110/110 cum laude) University of Milano-Bicocca, Milan (Italy).

November 2009- July 2011: Thesis in Developmental and Differentiation Genetics, Oncology (Prof. Silvia K. Nicolis, Dr. Ulrich Schüller "Functional role of the Sox2 transcription factor in cancer stem cells: a study by conditional knock-out of the Sox2 gene in mouse genetic models of neural tumors.")

10/2010 – 03/2011: Guest student (supported by ExTra Plus Program, awarded by Fondazione Cariplo Milano)

Centre for Neuropathology and Prion Research, Ludwig-Maximilians University, Munich (Germany), group of Dr. Ulrich Schüller. (Molecular basis of medulloblastoma; role of Sox2, β1-integrin, and of Shh- and Wnt pathways in medulloblastoma onset and progression.)

2005-2008: Laurea Triennale - Bachelor's Degree in Biological Sciences (110/110) University of Milano-Bicocca, Milan (Italy).

Thesis in Computational Biology (Dr. Maurizio Bruschi, "Molecular dynamics study of the interaction between p53 DNA-binding domain and DNA.")

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Publications

Articles/Reviews:

- **Pagella P.**, Schwab M., Mitsiadis T. “Nogo-A regulates stem cell differentiation and enamel formation during tooth development and regeneration” In preparation
- **Pagella P.***, Neto E.*, Miran S., Woloszyk A., Lamghari M., Mitsiadis T. “Investigation of the cross talk between sensory innervation and mesenchymal stem cells from different origins in a microfluidic co-culture environment” In preparation
- **Pagella P.***, Cantu’ C.*, Basler K., Mitsiadis T. “Differential requirement of β-catenin and Bcl9/9l in tooth development” In preparation
- **Pagella P.**, Miran S., Mitsiadis T. (2015). “Analysis of developing tooth germ innervation using microfluidic co-culture devices” *Journal of Visualized Experiments* In Press
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Book chapter:

- L. Jimenez-Rojo, Z. Granchi, A. Woloszyk, A. Filatova, **P.Pagella**, T. Mitsiadis. Regenerative dentistry: stem cells meet nanotechnology. (In book: Horizons in Clinical Nanomedicine,. Editors: Pan Stanford Publishing. Edition: 1st edition, 2013)

Posters/Abstracts:

- 11th Annual Swiss Stem Cell Network Meeting (SSCN, Basel, 29/06/2015). **P.Pagella**, E.Neto, S.Miran, M.Lamghari, T.Mitsiadis "Choosing partners: do nerves prefer dental pulp stem cells or bone marrow stromal cells?" Poster.
- 11th International Conference on Nanosciences & Nanotechnologies (Thessaloniki, Greece, 8-11/07/2014). **P.Pagella**, E. Neto, A. Woloszyk, M. Lamghari, T. Mitsiadis "A microfluidic co-culture system for the study of orofacial innervation". Poster selected for oral presentation.
- 10th Annual Swiss Stem Cell Network Meeting (SSCN, Geneva, 4/06/2014). **P. Pagella**, A. Woloszyk, E. Neto, M. Lamghari, T. Mitsiadis. "Interactions between trigeminal sensory innervation and mesenchymal stem cells of different origins". Poster
- Bioimaging 2013 – 2nd International Symposium in Applied Bioimaging: from molecules to man (Porto, Portugal, 3-4/10/2013). **P.Pagella**, ME. Schwab, T. Mitsiadis "The role of Nogo-A in orofacial development and regeneration". Poster.
- 11th Day of Clinical Research (2013, Zürich). **P. Pagella,** A. Woloszyk, E. Neto, M. Lamghari, T. Mitsiadis "Cross talk between sensory innervation and mesenchymal stem cells for orofacial regeneration in a microfluidics co-culture system". Poster
- 11th International Conference on Tooth Morphogenesis and Differentiation (TMD; La Londe les Maures, 26/05/13-31/05/13): **P.Pagella**, M. Alexiou, M. Schwab, T. Mitsiadis "The role of Nogo-A in orofacial development and regeneration". Poster.
- 9th Annual SSCN (Swiss Stem Cells Network) Meeting (Bern, 8/02/2013): **P.Pagella**, M. Alexiou, M.E. Schwab, T. Mitsiadis. "The role of Nogo-A in orofacial development and regeneration". Poster.
- Hydra VIII: European Summer School 2012 (Hydra, September 2012): A. Badiola Sanga, **P. Pagella**, I. Appolloni, R. Galli, P. Malatesta, S. Nicolis and R. Favaro. "A role for *sox2* in cancer stem cells? Studies by conditional *sox2* deletion in mouse models of neural cancers".
- EuroSystem Consortium Meeting (Prague, 6/06/11-10/06/11): R. Favaro R., **P. Pagella**, A. Badiola, F. Ebner, I. Appolloni, F. Calzolari, U. Schüller, P. Malatesta, S. Nicolis. "Functional role of Sox2 in neural cancer stem cells? An approach through conditional Sox2 deletion".

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2012: COST NAMABIO Training School

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Laboratory expertise

- PCR, Real Time PCR
- Cloning, plasmid design and preparation
- Transfections; lentivirus and retrovirus production and use
- Establishment and maintenance of primary cell cultures: neural stem cells, neurospheres, medulloblastoma neurosphere-forming cells, cerebellar granule neuron precursors, oligodendroglioma cell lines, sensory neurons, mesenchymal stem cells
- Organ cultures (tooth germs, dorsal root ganglia, trigeminal ganglia)
- Co-cultures in microfluidic systems
- Immunocytochemistry, immunohistochemistry, immunofluorescent stainings
- Widefield and confocal light microscopy
- Mouse genetic models
- Basic knowledge and practical experience on molecular dynamics

Teaching activities

- Teaching assistant: Grundlagenpraktikum Chemie – CHE 173 (Bachelor of Science UZH in Biology, 135 hours, Module leader: Prof. S. Bienz).
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Animal handling:	Felasa Category B Certificate	

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